The functionality of maternal and neonatal fatty acids : from pregnancy to childhood

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The studies presented in this thesis were performed within GROW - School for Oncology and Developmental Biology and within NUTRIM School for Nutrition, Toxicology and Metabolism which participates in the Graduate School VLAG (Food Technology, Agrobiotechnology, Nutrition and Health Sciences), accredited by the Royal Netherlands Academy of Arts and Sciences.

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Contents

Chapter 1

General introduction

Introduction

Essential fatty acids (EFAs) and their longer-chain more-unsaturated derivatives (the long-chain polyunsaturated fatty acids, abbreviated as LCPUFAs) are collectively named the essential PUFAs (ePUFAs) [1]. These fatty acids are integral components of all cell membranes, in which they play an important role in maintaining the structural and functional characteristics of those membranes. Furthermore, especially LCPUFAs may contribute to other life processes such as fetal brain development, fetal growth and the development of the immune system [2-4]. Since EFAs cannot be synthesized *de novo* by humans and the fetal LCPUFA synthesis from these EFAs is rather low [5], the fetuses depend on their mother's ePUFA consumption and metabolism as well as on the placental transfer of these fatty acids. This is indicated by the positive correlation between the maternal and neonatal LCPUFA status at birth [6-8]. Given that pregnancy is associated with a decrease in the LCPUFA status, this may have consequences for fetal development. Therefore, in this thesis, we present studies which are conducted to elucidate whether fetal exposure to certain LCPUFAs, reflected by maternal fatty acid concentrations during pregnancy and/or neonatal fatty acid levels at birth, are associated with fetal brain function (measured by fetal habituation), fetal growth and later immune function.

The lactation period is characterized by a decrease in the relative maternal docosahexaenoic acid (DHA, 22:6n-3) levels and supplementation with n-3 LCPUFAs may prevent this decline. However, an increased consumption of n-3 LCPUFAs may cause for a concomitant reduction of the required n-6 LCPUFAs and of arachidonic acid (AA, 20:4n-6) in particular [9]. Since breast milk contains AA and DHA, this reduction in AA may be unfavorable for breast-fed infants, because they also require an optimum supply of n-6 LCPUFAs. Accordingly, this may influence neonatal development and, therefore, we investigated the effect of n-3 and n-6 fatty acid supplementation on their concentrations in human milk.

The next paragraphs give relevant background information on fatty acids in general and the influence of pregnancy and lactation on ePUFA concentrations. Subsequently, some introductory information about fetal habituation, fetal growth, the humane immune system and their relation with fatty acids is given. Finally, an overview of the research questions will be presented.

Essential fatty acids and their long-chain polyunsaturated derivatives

Fatty acids are the building blocks for lipids and comprise a chain of carbon atoms (C-atoms) with at one end a methyl (CH_3) head group and at the other end a carboxyl (COOH) tail group, see **figure 1**.

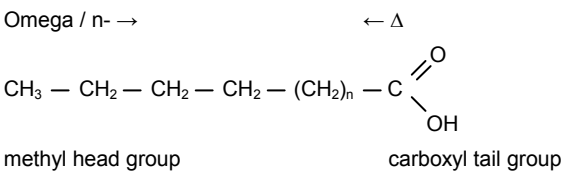

Figure 1. General structure of a saturated fatty acid

Fatty acids with no double bonds between the carbon atoms are named 'saturated', those with one or more double bonds are called mono- or polyunsaturated, respectively. According to the position of the first double bond (designated as 'omega' or 'n-'), unsaturated fatty acids can either belong to the n-3, n-6, n-7 or n-9 family. In these families the first double bond of the fatty acid is located between the 3rd and 4th, 6th and 7th, 7th and 8th or 9th and 10th carbon atom, as counted from the methyl head group, respectively. There are several notations to identify fatty acids. In this thesis, the first number of the notation refers to the number of C-atoms, the second number to the number of double bonds and the last number refers to the family assignment. For example: DHA, written as 22:6n-3, contains 22 C-atoms, 6 double bonds and belongs to the n-3 family.

Parent fatty acids containing 18 carbon atoms with the first double bond at the n-6 or n-3 position, cannot be synthesized endogenously from other components by humans. The reason is that humans lack the Δ12- and Δ15-desaturase enzymes, which enable the insertion of double bonds between the 6th and 7th, and 3rd and 4th carbon atom, respectively, counted from the methyl head group. Since these fatty acids are needed for several important body functions, they have to be obtained from the diet and are therefore called EFAs [10]. The parent EFAs of the n-6 and n-3 families are linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3), respectively. These EFAs are mainly present in seed oils (LA and ALA) and green leafs (mainly ALA). Both parent fatty acids can be further metabolized in the human body by the same series of desaturase and elongase enzymes to longer-chain more-unsaturated derivatives, the LCPUFAs (see **figure 2**).

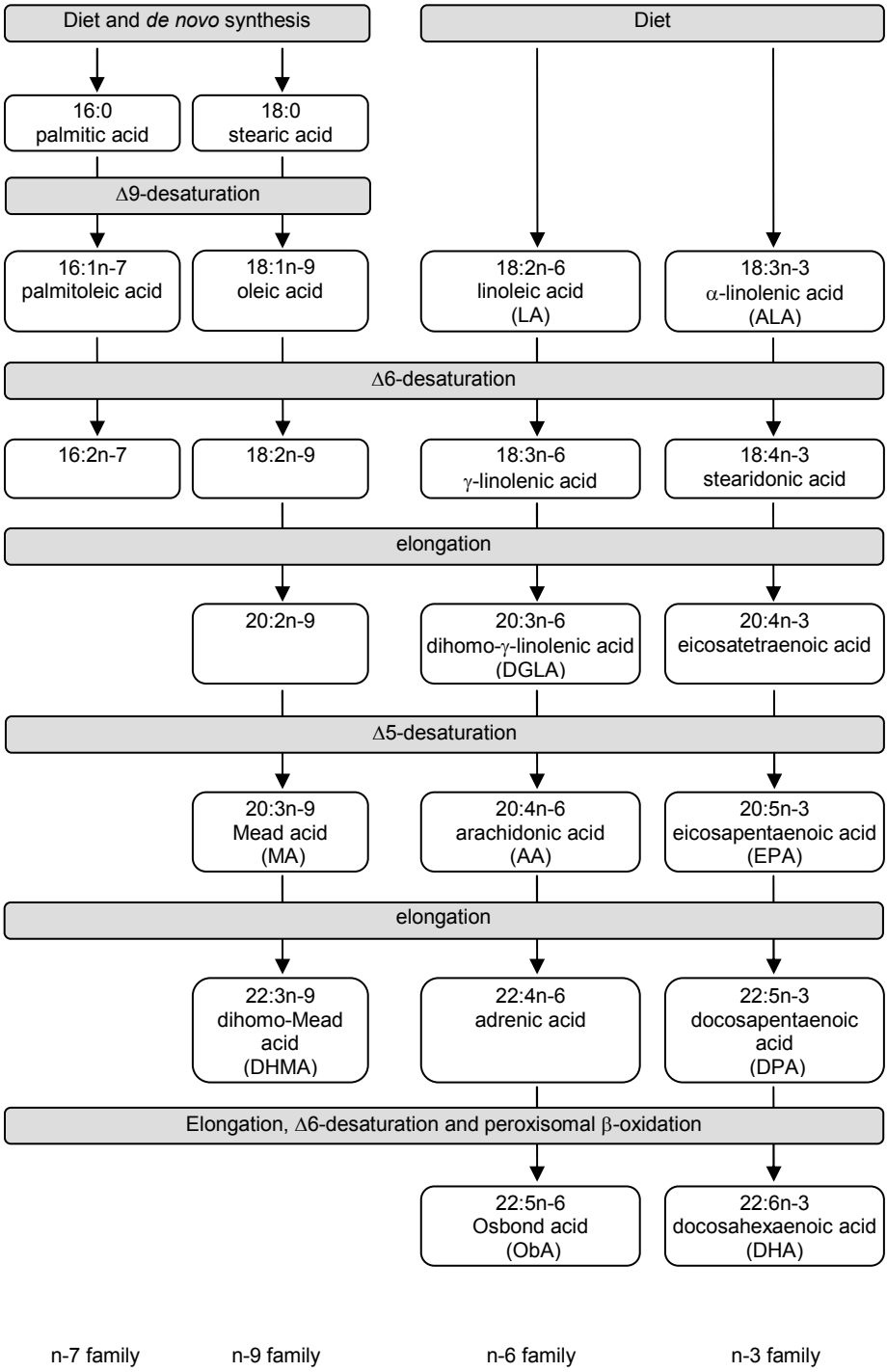

Figure 2. The main synthetic pathways of fatty acid metabolism

The desaturase enzymes only insert double bonds between the carboxyl tail group and the closest double bond and, consequently, do not affect the molecular structure at the methyl end of the fatty acid molecule. Therefore, all metabolized fatty acids remain in the same fatty acid family. The Δ6-desaturase enzyme, which inserts a double bond between the 6th and 7th carbon atom counted from the carboxyl tail of the chain, prefers ALA, followed by, in order, LA, oleic acid (18:1n-9) and palmitoleic acid (16:1n-7) [3]. The Δ6-desaturation is very important, since this step is considered to be the controlling step of the pathway [11]. Although the Δ6-desaturase has a preference for ALA, the abundant availability of LA compared to ALA in the present Western diet can be expected to promote LA conversion at the expense of ALA. As a result, only a little bit of ALA can be converted in eicosapentaenoic acid (EPA, 20:5n-3) (6.95 %) and DHA (0.08 %) [12,13]. When the LA and ALA levels are inadequate to meet the requirements, oleic acid will be metabolized to Mead acid (MA, 20:3n-9) and dihomo-Mead acid (DHMA, 22:3n-9). Because LCPUFAs inhibit MA and DHMA synthesis, the presence of MA and DHMA indicate a shortage of all ePUFAs and are, therefore, named ePUFA status markers [1]. There can also be a functional shortage of DHA. In that case, the body starts to synthesize the most comparable LCPUFA of the n-6 family, Osbond acid (ObA, 22:5n-6) [14,15]. However, evidence is becoming available that ObA may not always be a useful biochemical measure of a low DHA status [16].

Trans fatty acids

Trans fatty acids are unsaturated fatty acids with at least one double bond in the *trans* configuration. They occur naturally in dairy and other animal fats as a result of the biological hydrogenation of polyunsaturated fatty acids in the stomach of ruminants, but dietary *trans* fatty acids result mainly from industrial hydrogenation of edible oils. Foods with major contributions to *trans* fatty acid intake are baked goods such as doughnuts, margarines, imitation cheese and deep-fried foods like fried chicken and french-fried potatoes [17]. There is increasing evidence that industrially produced *trans* fatty acids promote ischemic heart disease. In addition, positive associations between the intake of these fatty acids and allergy, diabetes and cancer are reported [18]. As a result, the food industry responded by reducing the use of partially hydrogenated fats which resulted in a decrease in the consumption of *trans* fatty acids. Consequently, the intake of *trans* fatty acids in the Netherlands fell from 15 g per day in 1980 to 3 g per day in 2003 [19].

Positive correlations are observed between maternal and neonatal *trans* fatty acids [20,21]. Since the human fetus does not possess the function to produce *trans* fatty acids [22], the neonatal *trans* fatty acids originate from the maternal diet. Dietary *trans* unsaturated fatty acids have been shown to inhibit

the conversion of parent EFAs into their LCPUFAs, especially when the EFA contents are low [22,23]. They may also impair placental LCPUFA transfer [24]. Thus, *trans* fatty acids may lower the fetal LCPUFA status and thereby could compromise fetal development such as growth. This will be investigated in the present thesis.

The influence of pregnancy on maternal ePUFA concentrations

During pregnancy, accretion of maternal, placental and fetal tissue occurs. As a result, the mother adapts her metabolism in order to support the continuous draining of substrates, like n-3 and n-6 fatty acids, necessary for these changes [25]. A prospective longitudinal study of Al et al. showed that the total absolute amount (mg/L) of fatty acids in maternal plasma phospholipids (PLs), measured from early pregnancy towards the end of the pregnancy, increased by 51 % [26]. Similar patterns were found for the individual fatty acids and their fatty acid families. For example, the absolute amounts of DHA and AA increased by 52 % and 23 %, respectively. However, the proportional rise in absolute amounts of the non-essential unsaturated fatty acids (65 %) is considerably larger than the fractional increase in the absolute ePUFA amounts, which results in a significant decline of the EFA index [(n-3 + n-6)/(n-7 + n-9)] as pregnancy progresses.

Besides measuring fatty acids in absolute amounts, fatty acids can also be expressed as relative concentrations (% by wt of total amount of identified fatty acids). The relative fatty acid concentrations showed a different pattern as compared to the absolute amounts. Thus, as pregnancy progressed, the relative level of AA declined. The relative DHA levels increased between the 10^{th} and the 18^{th} week of pregnancy. After this period a gradual reduction was found, but DHA levels remained higher than prepregnancy levels throughout gestation. Nonetheless, the ObA synthesis, reflected by the ObA/adrenic acid (22:4n-6) ratio, increased gradually throughout pregnancy, which could indicate a reduction of the functional DHA status. These results suggest that the maternal ePUFA status declines during pregnancy.

After delivery, a slow normalization of the ePUFA status in maternal plasma PLs takes place in approximately 32 weeks [26,27].

The influence of lactation on maternal ePUFA concentrations

Human milk fat is the major source of energy for the infant, contributing 40-55 % of the total energy intake. The fat composition is influenced by factors such as maternal diet, duration of pregnancy and stage of lactation. The relative n-6 and n-3 LCPUFA concentrations of mature breast milk lipids from mothers

consuming a western diet is about 1.28 % and 0.48 % of the total amount of fatty acids, respectively. Furthermore, the relative AA and DHA concentrations in human milk are 0.45 % and 0.23 %, respectively [28]. The newborn infant is capable of synthesizing AA and DHA from precursor fatty acids, but this capacity seems insufficient to meet the high demands of the developing tissues [29,30]. Consequently, for these LCPUFAs, infants largely depend on an adequate dietary supply after birth, preferably from breast milk.

During lactation, women continue the transfer of their own LCPUFAs to their infants. The relative DHA concentrations in plasma PLs of lactating mothers, become lower than those of non-lactating mothers and even lower than those before conception [27]. Since DHA seems to be important for brain and retina development and function, this may have negative effects for the neonate. On the other hand, increasing the consumption of n-3 LCPUFAs may cause a concomitant reduction of circulating n-6 LCPUFA concentrations and of AA in particular [9]. This may be unfavorable in pregnant women and lactating women, because AA is considered essential for fetal and neonatal development [31,32]. Since data from supplementation studies are limited we studied the effect of n-3 LCPUFA supplementation, with or without AA, on AA and DHA levels in breast milk of lactating women.

Fetal habituation and the ePUFA status

In the womb the human fetus is surrounded by several acoustic stimuli, like the maternal intestinal and vascular noises. A fetus, just like an animal in the wildlife, needs to be able to distinguish those stimuli that are biologically normal and safe, like wind noises, from those stimuli that are potentially dangerous, like a growling lion. The fetus must learn to ignore the meaningless and safe stimuli in order to prevent needless anxiety. The ability to ignore this constant stimulation is provided by the process which is called habituation. Habituation is defined as the decrease in, and ultimate cessation of, the response to repeated stimulation with the same stimulus [33,34]. Furthermore, it is often considered to represent a form of learning and probably requires an intact and functioning central nervous system [35]. Memory, a prerequisite for fetal learning, is essential for normal functioning and it is likely that memory starts to develop during the prenatal period. In the beginning it probably functions in some rudimentary form and develops as the individual matures. Fetal memory may be important for the development of attachment and maternal recognition, for the establishment of breastfeeding, and for language acquisition [36]. Nowadays, it still is not known from what fetal age this memory can be established and for how long this memory lasts. Therefore, we investigated this in the present thesis.

Brain development is a complex process in which early disruptive events, like an inappropriate nutrients supply, can have long-lasting effects on later functional adaptations [37]. During periods of rapid brain growth, especially during the third trimester of pregnancy and the first months after birth, high accretion of both AA and DHA take place in the brain [2]. Therefore, these fatty acids are thought to be important for fetal brain development and brain function [37,38]. Since pregnancy is associated with a temporary decrease in the biochemical LCPUFA status of the mother, fetal brain function may not be optimal. Therefore, we investigated if some aspects of fetal brain function measured by fetal habituation, *viz*. fetal learning and memory, were related to the ePUFA status.

Potential consequences of the early LCPUFA status on fetal growth

The LCPUFAs DHA and AA are thought of critical importance for fetal brain development and fetal growth, respectively [2,3]. Because the maternal LCPUFA status declines during the course of pregnancy, the LCPUFA supply to the developing fetus may not always be optimal. Since more evidence is becoming available that low birth weight is associated with negative health outcomes later in life, it seems prudent to optimize fetal growth during pregnancy [39]. Several studies investigated the relationship between fetal growth and LCPUFAs, but results remain inconclusive [40-42]. Therefore, we investigated if there are associations between some specific birth dimensions and maternal and neonatal LCPUFAs measured during pregnancy and/or directly after delivery.

Arachidonic acid and the human immune system

The immune system consists of several organs and cell types that protect the host against pathogenic organisms. There are two types of immunity, the innate and the adaptive system. Innate immunity is present at all times in normal individuals and is thus fully functional before infectious agents enter the body. In this type of immunity phagocytes, including monocytes, macrophages and neutrophils, play an important functional role. In addition, the human body has the possibility to develop specific immunity against individual invading agents such as bacteria, viruses, toxins, etc. This is called the adaptive system, in which lymphocytes play an important role [43].

20-carbon LCPUFAs can serve as precursors for the synthesis of bioactive lipid mediators named eicosanoids. Eicosanoids include prostaglandins, thromboxanes, leukotrienes and other oxidized derivatives. Since immune cells contain high contents of AA, and low contents of other 20-carbon PUFAs, AA is usually the major substrate for prostaglandin E2 (PGE2), which is thought to be

an important mediator of immune responses [44,45]. As mentioned above, earlier studies revealed that the maternal AA status declines during pregnancy [26]. As a result, the AA supply to the developing fetus and its immune system may not always be optimal. In the literature, the relationship between prenatal AA concentrations and later immune-related variables remains relatively unexplored. Therefore, we investigated if prenatal AA exposure is related to several immune-related clinical conditions and inflammation markers of the child at seven years of age.

Research questions

The above presented literature underlines the importance of getting insight in the associations between EFAs and LCPUFAs and fetal learning and memory, growth and immune function. Also the effect of n-6 and n-3 fatty acid supplementation on breast milk levels is of importance. Therefore, in this thesis we will concentrate on the following research questions:

1) From which gestational age can fetal learning and memory be established, how long does fetal memory lasts and do fetal learning and memory depend on fetal age? (**Chapter 2**)

2) Are fetal learning and memory associated with the early essential polyunsaturated fatty acid status? (**Chapter 3**)

3) Are neonatal birth dimensions associated with maternal essential and *trans* fatty acid contents, sampled during pregnancy and at delivery? (**Chapter 4**)

4) Are neonatal birth dimensions associated with prenatal exposure to essential and *trans* fatty acids? (**Chapter 5**)

5) What is the effect of n-3 LCPUFA supplementation, with or without AA, on AA and DHA levels in breast milk of lactating women? (**Chapter 6**)

6) Is prenatal AA exposure associated with immune-related clinical conditions and plasma markers in childhood? (**Chapter 7**)

In **chapter 8**, the main results of these studies and their possible implications are discussed.

Chapter 2

Aspects of fetal learning and memory

Chantal E.H. Dirix, Jan G. Nijhuis, Henk W. Jongsma and Gerard Hornstra

Based on: Child Development (in press)

Abstract

Ninety-three pregnant women were recruited to assess fetal learning and memory, based on habituation to repeated vibroacoustic stimulation of fetuses of 30-38 weeks gestational age (GA). Each habituation test was repeated 10 minutes later to estimate fetal short-term memory. For groups 30-36, both measurements were replicated in a second session at GA 38 for the assessment of fetal long-term memory. Within the time frame considered, fetal learning appeared GA-independent. Furthermore, we observed that fetuses have a short-term (10 minutes) memory from at least 30 weeks GA onwards, which also appeared independent of fetal age. In addition, results indicated that 34 week old fetuses are able to store information and retrieve it 4 weeks later.

Introduction

Habituation is the decrement in attention or response following repeated stimulation with the same stimulus [33]. This phenomenon can be distinguished from phenomena such as effector fatigue or receptor adaptation since it requires an immediate recovery of the response on presentation of a different stimulus and it also requires that the response decrement occurs faster upon re-presentation of the original stimulus [33,35]. The first study of fetal habituation was reported in 1925, in which a decrease in fetal movements was observed after repeated stimulation with a car horn [46]. Since then several studies have shown that repeated stimulation of a fetus with the same stimulus, such as an electric toothbrush [47], a vibroacoustic stimulator [48] or a door buzzer [49], resulted in a decrement of its response. The decrement of the fetal response was assessed by, for example, changes in fetal heart rate [50] or fetal movements [51].

Habituation is considered to represent a form of learning and probably requires an intact and functioning central nervous system (CNS) [35]. A better understanding of the normal development of the fetal CNS will lead to more insight into abnormalities, allowing, prevention and/or extra care in the first years of life and, as a consequence, to less problems in later life. Fetuses whose behavioral development lags behind need significantly more stimuli before habituation occurred than well-developed fetuses of the same gestational age (GA). This was demonstrated by Morokuma et al., who defined three behavioral indicators to classify the developmental stage of fetal CNS function: 1) alteration of the eye movement and no eye movement periods, 2) rapid and slow eye movement patterns and 3) concurrence of regular mouthing movement in the no eye movement period [51]. Impaired habituation performance has also been identified in fetuses diagnosed with Down's syndrome. In the study by Hepper and Shahidullah, one fetus failed to habituate completely and the other habituated more slowly than the normal fetuses tested [52].

It is likely that memory starts to develop during the prenatal period. In the beginning it probably functions in some rudimentary form and develops as the individual matures. Fetal memory may be important for the development of attachment and maternal recognition, for the establishment of breastfeeding and for language acquisition [36]. Memory can be divided in a short-term memory, which lasts seconds till a couple of hours, a long-term memory, which may lasts hours to months and a long-lasting memory which may last a life-time [53]. Van Heteren et al. used fetal habituation to assess fetal memory [48,54]. Repeated vibroacoustic stimuli of 1 second each were applied every 30 seconds and a general movement of the fetal trunk within 1 second of application was defined as a positive response. Cessation of the fetal response movements for 4 consecutive stimuli was taken to indicate habituation. Using this methodology, these authors observed that a short-term memory of at least

10 minutes is present in normal healthy term fetuses, which may even last for 24 hours.

Several investigators studied fetal habituation in relation to post-conceptional age with inconsistent results. Morokuma et al., for example, defining habituation as the gradual and ultimately complete extinction of the fetal movement response for five consecutive vibroacoustic stimuli, observed that the number of stimuli to require habituation was significantly and inversely related to GA [51]. Groome and coworkers scored the intensity of the fetal response to each stimulus and they classified the fetal response as: fast general (a rapid, intense full-body startle response), fast local (a fast head or limb movement), slow roll (slow turning of fetus, usually confined to slow movement of the head and/or trunk) and no response [55]. Repeated vibroacoustic stimuli were given for 2 seconds each, separated by 5-second off periods. In total, a maximum of 14 stimuli were presented and they also found that older fetuses habituated significantly faster than younger ones. Madison and coworkers on the other hand could not find a significant correlation between fetal age and the rate of habituation [56]. However, they used a vibrator to stimulate the fetuses and defined habituation as extinction of the fetal response for five consecutive stimulations. Consequently, it still is not known from what post-conceptional age a fetal memory can be ascertained and for how long a fetus can recognize a specific stimulus. Therefore, the purpose of the present study was to investigate from which gestational age fetal learning and memory can be established, how long fetal memory lasts and whether fetal learning and memory depend on fetal age.

Method

Study population

The population of the habituation study included 100 healthy Dutch Caucasian pregnant women who were recruited through midwives in the Southern Limburg region of The Netherlands and the Department of Obstetrics and Gynaecology of the Maastricht University Medical Centre (MUMC) in Maastricht. The study was approved by the Ethics Committee of the MUMC and all mothers gave their informed consent in writing.

Inclusion criteria at intake were: (a) gestational age between 28-38 completed weeks (gestational age was determined using the last menstrual period or by ultrasound when dates were uncertain); (b) absence of maternal metabolic, cardiovascular, renal or neurological disorders; (c) no use of medication (iron and folate allowed), drugs or alcohol (more than 7 glasses per week); (d) smoking less than 6 cigarettes per day; (e) no change in nutritional habits during pregnancy; (f) no depression during the present pregnancy or not

more than one episode of depression before the present pregnancy; (g) a diastolic blood pressure below 90 mm Hg; (h) single fetus without apparent structural anomalies.

Exclusion criteria during the study were: (i) parturition before week 37 or after week 43 of gestation; (j) fetus with a birth weight below the 10th percentile according to population-based tables adjusted for pregnancy duration and fetal sex [57]; (k) abnormal amniotic fluid volume as assessed by ultrasound.

Maternal and neonatal characteristics

At the start of the study, a basal (intake) questionnaire was filled out by all volunteers, which included the following items: maternal age, pre-pregnancy weight, height, weight and height of the father, medical history of the mother including former pregnancies, maternal consumption of fish and nutrient supplements, smoking and drinking habits of the mother and choice of infant feeding. Education was scored on an 8-point scale, ranging from primary education to higher vocational training and university [58]. The outcome of each pregnancy was examined in terms of mode of delivery, birth weight, Apgar scores, infant sex, gestational age at delivery and the presence of neonatal abnormalities.

Fetal habituation method

Fetal habituation was assessed according to the protocol used by Van Heteren and coworkers [48,59]. All habituation tests were performed by the same examiner (C.E.H. D.), between 5 and 8 pm in a darkened quiet room. The volunteers were placed in a semi-recumbent position and were not allowed to drink coffee or tea, smoke and eat for 3 hours before testing.

Every 30 seconds, a vibroacoustic stimulus (VAS) of 1 second was applied to the maternal abdomen above the fetal legs, using a fetal vibroacoustic stimulator (Corometrics model 146, Wallingford, CT, USA; audible sound 20-9000 Hz, vibrations 67-83 Hz, sound level 74 dB at 1 m in air). Movements of the fetus within 1 second after application of the stimulus and monitored by an ultrasound scanner (HDI-5000, Bothell, USA) displaying the fetal trunk, was considered a positive response. VAS-elicited fetal responses are typical immediate movements which can easily be distinguished from a coincidental movement by the mother. Therefore, fetal movements experienced by the mother within 1 second after application of the stimulus, but missed by the scanner, were accepted as a positive response also. However, in the vast majority of the tests, movements were readily picked up by the scanner. Disappearance of the response for four consecutive stimuli was taken to demonstrate habituation. To prevent interference by spontaneous fetal movements, habituation tests started after a three-minutes period in which the

fetus was not moving spontaneously [60]. After fetal habituation was identified, stimulation was stopped. There was a maximum of 24 stimulus applications in each test. However, when a fetus was still responding to the 21st stimulus, a minimum of 4 extra stimuli would be necessary to determine habituation. Therefore, no further stimuli were given if a fetus still responded to the 21st stimulus. The habituation rate was defined as the number of consecutive stimuli applied before habituation was established. This is considered a measure of the fetal learning capacity at the moment of the habituation measurement. Habituation tests, in which fetuses reacted inconsistently to the VAS so that habituation could not be established, were considered missing and as a consequence these data were ignored in calculating and analyzing the results. Fetuses were excluded if they did not respond to VAS at the initial habituation test.

Measuring scheme and calculations of fetal learning and memory

Habituation was measured several times in each fetus, as explained below and outlined in **table 1**.

Table 1. Schedule of habituation measurements per group[a]

	First session								Second session			
Group	GA 30		GA 32		GA 34		GA 36		GA 38			
	A	B	A	B	A	B	A	B	A	B	C	D
30	HR	HR									HR	HR
32			HR	HR							HR	HR
34					HR	HR					HR	HR
36							HR	HR			HR	HR
38									HR	HR	Not applicable	

[a] HR measurement = measurement of habituation rate; GA = gestational age in weeks; A = initial habituation rate measurement at first session; B = repetition, 10 minutes after A; C = initial measurement at second session; D = repetition, 10 minutes after C.

Habituation rate (fetal learning) as function of gestational age

Depending on their pregnancy durations, volunteers were divided over five groups. Fetal habituation rates (HR) were measured at two times during a first test session at GA of 30 (group 30), 32 (group 32), 34 (group 34), 36 (group 36) or 38 weeks (group 38). For groups 30-36, these two measurements were

repeated during a second test session at GA 38. The values of the first habituation rate measurement (HR-A) were considered a measure of fetal learning and were correlated to GA as described in data analysis.

Fetal 10-minute memory

The difference in habituation rates (HR) of a given fetus between the two tests in each session is taken as a reflection of its short-term (10 minutes) memory and is expressed as a percentage of the initial habituation rate. After the initial habituation test of the first session at 30, 32, 34, 36 or 38 weeks of gestation (HR-A, see **table 1**), all fetuses were tested again in exactly the same way 10 minutes later (HR-B). The fetal 10-minute memory during these first test sessions (STM-1) was calculated as 100*[(HR-A) minus (HR-B)]/(HR-A). At GA 38, habituation rates of the fetuses of groups 30-36 were again measured twice with a 10 minutes interval (HR-C and HR-D, respectively) and the 10-minute memory during this second session (STM-2) was calculated as 100*[(HR-C) minus (HR-D)]/(HR-C). However, if fetuses did not respond to the initial VAS stimulus at the second session (HR-C = 0), percentage calculation would require division by zero, which is not possible. Therefore, 0.5 was added to all HR values measured.

Fetal long-term memory

The habituation rates measured in fetuses of groups 30-36 at 38 weeks GA may, at least partly, result from 'memorizing' the earlier habituation measurements at GA 30-36. If this is the case, their HR values at GA 38 (HR-C) will be significantly lower than their HR values at 30-36 weeks GA (HR-A) and they will also be significantly lower than those of 38 weeks old fetuses who did not experience an earlier habituation test before (Group 38). Therefore, to assess the presence of a long-term memory in 38 week old fetuses, HR-C values of the fetuses of groups 30-36 were statistically compared to their own HR-A values. Finally, in the groups in which this comparison demonstrated significance (indicating the presence of a long-term memory indeed), these HR-C values were statistically compared to the HR-A values of group 38. This comparison was not performed in groups with no significant differences between HR-C and HR-A values, since this outcome excludes the presence of a long-term memory.

Data analysis

All data were checked for normality by histograms and Shapiro's test. Since most data were not normally distributed, non-parametric statistics were applied for data evaluation.

Differences in maternal and neonatal characteristics between the five groups were tested for significance with either the Chi-Square test (discrete variables) or the Kruskal-Wallis test (continuous variables). Differences between paired data were evaluated with the Wilcoxon signed-rank test and distinctions between unpaired data were analyzed with the Mann-Whitney U test.

Relations between two variables were assessed with the Spearman's rank correlation test. Application of the various tests will be mentioned when describing the results.

For all statistical analyses, a p-value < 0.050 was considered statistically significant. All statistical analyses were performed using the statistical package SPSS 11.5 for Windows (release 11.5, SPSS Inc., Chicago, Illinois).

Results

Eleven women who were included in the study dropped out for various reasons: (a) one volunteer delivered (almost 24 hours after rupture of the membranes) her baby before the planned test date at 38 weeks GA and her baby died one day after birth due to a streptococcal infection; (b) two mothers had pregnancy-induced hypertension; (c) one volunteer stopped because her fetus developed intra-uterine growth retardation; (d) one child was born before 37 weeks GA; (e) one child had a birth weight below the 10^{th} percentile and (f) five volunteers were excluded because their fetuses did not react to the VAS at the initial habituation test of the first session (HR-A). Women who were excluded during the study were replaced by new volunteers. However, due to time restrictions, seven volunteers could not be replaced, leaving 93 volunteers instead of 100. Therefore, group 30 included 17 volunteers and groups 32 and 38 consisted of 18 volunteers instead of 20.

The relevant maternal and neonatal characteristics are displayed in **table 2**. Groups did not differ significantly in maternal age, height, pre-pregnancy body mass index (BMI), educational level, parity and mode of delivery (p = 0.189 - 0.991; Kruskal-Wallis test). Differences in neonatal weight, gestational age at delivery and Apgar score at 5 minutes (p = 0.301 - 0.909; Kruskal-Wallis test) were also not significant between the groups. The same holds for infant sex, although there was a trend for a higher number of female fetuses (p = 0.052; Chi-Square test). All included neonates were in good health after birth, with a 5-minute Apgar score ≥ 8 and a birth weight > 10^{th} percentile and no congenital anomalies were detected.

Table 2. Maternal and infant characteristics[b]

Characteristics	Group 30	Group 32	Group 34	Group 36	Group 38
Maternal characteristics					
Number of volunteers	17	18	20	20	18
Age (years)	31.0 (27.0 - 35.0)	30.5 (28.5 - 32.0)	31.0 (28.0 - 32.8)	33.5 (29.0 - 35.0)	29.5 (27.5 - 32.3)
Height (m)	1.68 (1.65 -1.76)	1.68 (1.63 - 1.73)	1.70 (1.68 - 1.76)	1.68 (1.66 - 1.73)	1.70 (1.66 - 1.74)
Pre-pregnancy BMI (kg/m^2)	23.9 (22.4 - 26.1)	23.6 (21.4 - 25.2)	21.6 (20.4 - 24.1)	21.7 (20.3 - 23.5)	22.6 (20.9 - 24.1)
Educational level	6.0 (4.0 - 8.0)	6.0 (4.0 - 6.5)	6.0 (4.3 - 7.5)	5.0 (4.0 - 7.5)	6.0 (4.0 - 8.0)
Parity at entry (n) 0/1/2/3	7/5/5/0	13/4/1/0	11/7/1/1	10/6/3/1	9/7/2/0
Mode of delivery					
Vaginally	15	11	14	17	13
Extraction (forceps / vacuum)	1	6	5	2	1
Caesarean section	1	1	1	1	4
Infant characteristics					
Birth weight (g)	3305 (3065 - 3965)	3520 (3020 - 3661)	3613 (3061 - 4070)	3560 (3098 - 4333)	3630 (3320 - 3825)
Gestational age at delivery (weeks)	40.0 (39.2 - 41.4)	40.0 (39.4 - 40.5)	40.1 (38.5 - 40.5)	40.2 (39.3 - 41.1)	40.4 (39.9 - 41.0)
Sex (n) male / female	6 / 11	11 / 7	6 / 14	4 / 16	10 /8
Apgar score after 5 min	10 (10 - 10)	10 (10 - 10)	10 (9 - 10)	10 (10 - 10)	10 (10 - 10)

[b] Data are given as median and interquartile ranges (IQR), unless otherwise mentioned.

Subjects with incomplete data were nevertheless included. As a consequence, not all data analyses were based on the same number of subjects. In the vast majority of the habituation tests (95.2 %) the presence or absence of habituation could reliably be established. Only in 16 out of the 336 tests this appeared impossible, due to irregular responses (4 tests) or birth before the planned test date (12 tests). These 16 habituation tests concerned nine fetuses which did not differ from the others for the above mentioned maternal and neonatal clinical characteristics ($p = 0.092$ - 0.784; Mann-Whitney U test). The discrete variable infant sex was also not significantly different ($p = 0.677$; Chi-Square test). However, as expected, a significant difference existed in gestational age ($p = 0.001$; Mann-Whitney U test) due to the fetuses who were born before the planned test date. The maternal and neonatal characteristics of the fetuses ($n = 5$) who were excluded due to non-responses at HR-A were comparable with those included in the statistical analyses ($p = 0.209$ - 0.891; Mann-Whitney U test); this also accounts for infant sex ($p = 0.992$; Chi-Square test). The results of all habituation measurements are given in **table 3**.

Habituation rate (fetal learning) as function of gestational age

At 30 weeks GA, almost all fetuses demonstrated habituation already and no significant relation was observed between fetal learning, as reflected by the first measured HR values (HR-A) of all five groups, and gestational age at measurement (Spearman's rho = 0.052; $p = 0.622$).

Fetal 10-minute memory

In each group, with the exception of group 32, a significant decline was observed between the initial (HR-A) and repeated (HR-B) habituation tests in the first session (Wilcoxon signed-rank test; $p = 0.001$, $p = 0.305$, $p < 0.001$, $p < 0.001$, $p = 0.001$, for groups 30-38 respectively). Furthermore, there was a significant decrease in each group between HR-C and HR-D values of the second session at GA 38 ($p = 0.012$, $p = 0.001$, $p = 0.002$, $p < 0.001$, for groups 30-36 respectively). The 10-minute memory values (short-term memory, STM) were quantified as described above and results are reported in **table 4**. No significant correlation was observed between fetal age at the time of measurement and STM of the first session (STM-1, Spearman's rho = 0.042, $p = 0.692$). Between the STM of the first (STM-1, GA between 30 and 36 weeks) and second session (STM-2, GA 38 weeks) no significant differences were found for any of the groups ($p = 0.069$ - 0.983; Wilcoxon signed-rank test). Also, no significant difference was found between STM-2 of groups 30-36 taken together and STM-1 of group 38 (Mann-Whitney U test; $p = 0.590$). When the

Table 3. Fetal habituation rates[c]

Group	First session (GA 30 - 38, as indicated by group name)				Second session (GA = 38 wks)			
	Initial test (HR-A)		Repeated test (HR-B)		Initial test (HR-C)		Repeated test (HR-D)	
	n	Median (IQR)	n	Median (IQR)	n	Median (IQR)	n	Median (IQR)
30	16	13.0 (7.0 - 17.8)	17	2.0 (1.0 - 5.0)	16	6.5 (3.3 - 9.8)	16	1.0 (0.0 - 6.5)
32	18	5.0 (4.0 - 7.0)	17	1.0 (0.5 - 10.5)	16	4.0 (1.5 - 9.5)	16	0.0 (0.0 - 1.0)
34	20	10.5 (8.3 - 15.0)	20	2.0 (0.3 - 4.8)	17	5.0 (2.0 - 11.0)	16	0.0 (0.0 - 2.0)
36	20	11.0 (6.8 - 17.5)	20	3.5 (0.0 - 5.8)	20	5.0 (3.0 - 18.0)	19	1.0 (0.0 - 2.0)
38	18	11.0 (3.8 - 18.5)	18	1.0 (0.0 - 5.3)	Not applicable			

[c] Median habituation rates and their interquartile ranges (IQR) are presented per group per habituation test. GA = gestational age in weeks at measurement; HR = habituation rate. A rate of 0 indicates a non-response.

STM-2 values of groups 30-36 were tested separately against the STM-1 values of group 38, also no significant differences were found (Mann-Whitney U test; p > 0.384).

Table 4. Short-term (10-minutes) memory during both sessions[d]

Group	Session 1 (GA shown by group)		Session 2 (GA 38 week)	
	n	Median (IQR)	n	Median (IQR)
30	16	78.3 (46.8 - 85.7)	16	54.4 (0.0 - 92.0)
32	17	72.7 (-33.3 - 84.4)	16	73.8 (37.3 - 88.9)
34	20	76.6 (62.2 - 91.4)	16	76.2 (12.7 - 94.2)
36	20	75.2 (28.5 - 92.2)	19	70.6 (40.0 - 90.9)
38	18	69.5 (37.1 - 94.5)	Not applicable	

[d] Short-term (10-minutes) memory, given as median and interquartile ranges (IQR), is the decrease between two successive habituation rates measured with a 10 minutes interval and is expressed as a % of the initial habituation rate. GA = gestational age in weeks at measurement.

Fetal long-term memory

HR-C values of groups 30-36 were invariably lower than their HR-A values (see **table 3**), although these differences were significant for groups 34 and 36 only (p = 0.017 and p = 0.044, respectively; Wilcoxon signed-rank test). When HR-A results of group 38 were tested against HR-C values of the two groups separately (Mann-Whitney U test), p-values of 0.053 (group 34) and 0.331 (group 36) were found. These non-significant results might be due to the rather limited power and, therefore, HR-C values of groups 34 and 36 were taken together and compared to the HR-A values of group 38 by means of the Mann-Whitney U test. In this way it was evaluated whether there are significant differences between earlier tested fetuses and fetuses who had never been tested before. It then appeared that the habituation rates of earlier tested fetuses tended to be somewhat lower than those of fetuses of the same GA but tested for the first time. However, the differences were not significant (p = 0.095; Mann-Whitney U test).

Discussion

The aim of this study was to determine from which gestational age fetal learning and memory could be established, how long this memory lasts in utero and whether fetal learning and memory depend on gestational age. Our results showed that fetal learning, as reflected by the first habituation rate outcomes between the age of 30 and 38 weeks, is independent of GA. Furthermore, it was found that fetuses have a short-term (10 minutes) memory from 30 weeks GA onwards, which also seems independent of gestational age when it is measured for the first time. A long-term memory of 4 weeks is possibly present in 38 week old fetuses.

Habituation rate (fetal learning) as function of gestational age

Groome et al. found that older fetuses (36-40 weeks GA) habituated significantly faster than younger ones (28-32 weeks GA) [55]. A study of Morokuma et al. also showed that the number of stimuli to require habituation was significantly and inversely related to gestational age [51]. On the other hand, Madison et al. studied 39 fetuses between 28 and 37 weeks of gestation and could not find a significant correlation between fetal age and the rate of habituation, which is in line with our results [56]. Due to different methodologies used to assess habituation, it is difficult to point out reasons for these discrepancies. Although Groome et al. as well as Morokuma et al. used the same vibroacoustic stimulator as we did, these two studies differed from each other and ours in the maximum number of stimulations applied in each session. Groome et al. and we used maximally 14 and 24 stimuli, respectively, while Morokuma et al. did not define a maximum number of stimuli. Furthermore, Morokuma et al. included the data of only 26 volunteers in the study, whereas the study of Groome et al. and our own study enclosed 90 and 93 volunteers, respectively. Moreover, Groome et al. always gave 14 stimulations to each fetus while Morokuma et al. and we accepted a lack of response to five, respectively, four consecutive stimuli as habituation. Finally, the study of Madison et al. also differed in the aspects mentioned above and, moreover, their stimulator provided only vibrations and no acoustics.

In our study, the fetuses tested at 32 weeks GA showed the lowest HR-A values of all groups, which might suggest that fetal brain maturation reached an optimum for the habituation reaction already or that other undefined factors could have impacted the HR-values. For these suggestions to be acceptable, however, the HR-A values of groups 34-38 would need to be of the same order of magnitude, whereas they were comparable to the value observed for group 30. So it seems that the low HR-A values of group 32 happened by chance.

For a proper interpretation of our results it is essential to know whether the response decrement we observed is really due to habituation and not to effector

fatigue or receptor adaptation. Habituation can be distinguished from adaptation or fatigue by the recovery of a habituated response on presentation of a new stimulus (dishabituation) and faster habituation upon re-presentation of the original stimulus [33,35]. Although we *did* see faster habituation to the original stimulus in the 10-minutes test series (HR-B and HR-D), a limitation of the current study is that we did not present a novel (dishabituation) stimulus in the habituation series to exclude effector fatigue or receptor adaptation. However, since these phenomena have not been observed in comparable fetuses tested at a gestational age similar to that of the fetuses in our study and using stimuli largely comparable to the ones we applied [52,61,62], it seems unlikely that effector fatigue or receptor adaptation contributed to the response decrement we observed, but we cannot rule it out completely.

Fetal 10-minute memory

The presence of a short-term memory of ten minutes confirms the results of Van Heteren et al., who showed that, after an initial habituation test, fetuses between 37-40 completed weeks GA needed less stimuli for habituation 10 minutes later [48,54]. Hepper and Shahidullah also found a short-term memory in fetuses measured between 34-36 weeks GA [52]. The present study showed that the 10-minute memory, measured at the first session, was not dependent on fetal age from 30 weeks GA on. This implies that a 10-minute memory could already be present before week 30 of pregnancy. Although the earliest habituation response of a fetus to an auditory stimulus was noted at a post-conceptional age of 22-23 weeks [63], previous studies showed that only a minority of the fetuses is able to respond to VAS before the 29th week of pregnancy [64,65]. As a consequence, fetal memory might be present in an earlier stage of pregnancy, but this cannot be verified with this method.

Fetal long-term memory

In the present study, paired analyses in each group demonstrated a decline in habituation rate between the initial tests of the first and second session (HR-A versus HR-C), which suggests the presence of a long-term memory. Since these decreases were significant for groups 34 and 36 only, our observation suggests that a fetus of 34 weeks GA is able to store information and retrieve it 4 weeks later and, consequently, may have a 4-week's memory already at 38 weeks GA. However, in the unpaired comparison between HR-C tests of groups 34 and 36 taken together (measured at GA 38, fetuses were exposed to VAS before) and the HR-A test of group 38 (measured at GA 38 as well, but first VAS experience of the fetuses), the habituation rates were not significantly different. Since the p-value of this comparison ($p = 0.095$) indicates a trend, this outcome may point to the presence of a long-term memory indeed. It should be

recalled that unpaired statistics usually require more cases for a given difference to reach significance than paired analyses. Consequently, the power of the study may have been too low for this latter comparison. Under the presumption that adding more cases would not change the distribution of the habituation rate outcomes, post hoc power calculations showed that 40 % more cases per group would be required for significance of the present difference between the HR-C tests of group 34 and 36 and the HR-A test of group 38. In the literature, only one study used habituation to assess the presence of a long-term memory and found that habituation rates of ten fetuses between 34-36 weeks GA were not significantly different when they were measured again 7 days later [52]. Our results, nonetheless, indicate that a 4-week memory may exist in fetuses of 38 weeks GA, but further research is required for confirmation.

In conclusion, we observed that fetal learning and short-term (10 minutes) memory, as assessed by repeated fetal habituation, is present at 30 weeks GA already. In addition, we present some evidence that 34 week old fetuses are able to store information and retrieve it 4 weeks later.

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Chapter 3

Fetal learning and memory: Weak associations with the early essential polyunsaturated fatty acid status

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Abstract

To study the potential associations between fetal brain functions and the early essential polyunsaturated fatty acid (ePUFA) status, fetal learning and memory were assessed by repeated habituation rate measurements (HR) in fetuses of 30, 32, 34 or 36 weeks gestational age (GA). HR tests were repeated 10 minutes later. Both measurements were replicated in a second session at GA 38. Fetal short-term memory (STM) and long-term memory (LTM) were calculated from these habituation rates and related to concentrations of ePUFAs and their status markers, measured in umbilical artery wall phospholipids. The only relevant associations observed were positive trends ($0.010 < p < 0.050$) between STM measured before 38 weeks GA and levels of the ePUFA status markers Mead acid and Mead acid+dihomo-Mead acid, and between LTM and levels of Osbond acid, a marker of the n-3 LCPUFA status. Although these weak associations may imply some negative relationships between fetal brain functions and the early ePUFA status, we concluded that physiological differences in the availability of these fatty acids may probably not determine the differences in these primitive brain functions during the third trimester of fetal development.

Introduction

The essential long-chain polyunsaturated fatty acids (LCPUFAs), arachidonic acid (20:4n-6, AA) and docosahexaenoic acid (22:6n-3, DHA), are considered of great importance for brain development and function [37,38]. During pregnancy, the last trimester is noted for rapid development of the fetal brain and high accretion rates of AA and DHA [66]. To obtain these LCPUFAs, the fetus depends primarily on the placental transfer, and thus on the maternal supply, of these fatty acids [26]. However, since pregnancy is associated with a reversal decrease in the LCPUFA status of the mother, the fetal LCPUFA status may not be optimal [1], which may have consequences for the development of the fetal brain. We, therefore, assessed fetal brain functions and related these to the fetal exposure to essential fatty acids (EFAs) and their LCPUFAs, collectively called essential polyunsaturated fatty acids (ePUFAs) [1]. These fatty acids and their status markers were measured in the phospholipids (PLs) of umbilical cord artery walls. To assess fetal brain function, we measured fetal habituation, which is a non-invasive method to test the integrity of the fetal central nervous system functions [47] and can also be used to assess fetal memory [48]. Fetal habituation is the decrease in, and ultimate cessation of, a fetal response to repeated stimulation. It is considered to represent a form of learning and requires an intact and functioning central nervous system [35].

Patients and methods

Study design and population

Learning capacity and memory performance of fetuses were derived from habituation data, available from a previous study [67]. By means of unadjusted and multivariable-adjusted regression analyses, these early brain functions were related to the ePUFA status of the fetuses as reflected by selected fatty acid concentrations measured in the arterial wall PLs of their umbilical cords collected directly after delivery. The study was approved by the Ethics Committee of the Maastricht University Medical Centre and all included mothers gave their written informed consent. Initially, 5 groups of 20 women were included. Pregnancy duration at the start of the habituation measurements was 30-38 weeks. Details of in- and exclusion for the habituation data have been published before [67]. In addition, we excluded volunteers if the umbilical cord could not be collected at birth.

Fetal habituation method

All habituation tests were performed by the same examiner (C.E.H. D.) as described before [67]. Briefly, the fetal trunk was visualized by an ultrasound scanner and every 30 seconds a vibroacoustic stimulus (VAS) of 1 second duration was applied to the maternal abdomen above the fetal legs. A general movement of the fetus within 1 second of application of the stimulus was considered a positive response. A lack of response to 4 consecutive stimuli was taken to indicate habituation. We allowed a maximum of 24 stimuli in each habituation test and when fetal habituation was identified, stimulation was stopped. The habituation rate (HR) was defined as the number of consecutive stimuli applied before a fetus stopped responding. Habituation tests, in which fetuses reacted inconsistently to the VAS so that habituation could not be established, were considered missing and as a consequence these data were ignored in calculating and analyzing the results. Mothers were excluded if their fetuses did not respond to VAS at the initial habituation test and replaced by other volunteers of similar pregnancy durations.

Habituation protocol and fetal learning and memory calculations

Fetal habituation rates were measured for the first time (HR-A) during a session at gestational ages (GA) of 30 (group 30), 32 (group 32), 34 (group 34) or 36 weeks (group 36) and tests were repeated 10 minutes later (HR-B). Gestational age was determined using the last menstrual period or by ultrasound when dates were uncertain. Both measurements were replicated under the same conditions during a second test session at GA 38 (HR-C and HR-D).

The first habituation test outcome of each fetus (HR-A) was taken to reflect its learning capacity. The difference in habituation rates of a given fetus between the two tests in each session was regarded a reflection of its short-term (10 minutes) memory and expressed as a percentage of the initial habituation rate. Thus, fetal short-term memory during the first test session (STM-1) was calculated as 100*[(HR-A minus HR-B)/HR-A]. The short-term (10 minutes) memory during the second session (STM-2) was calculated as 100*[(HR-C minus HR-D)/HR-C]. However, if fetuses did not respond to the initial VAS stimulus at the second session (HR-C = 0), percentage calculation would require division by zero, which is not possible. Therefore, 0.5 was added to all HR values measured to calculate STM-1 and STM-2.

As mentioned before, habituation of the fetuses of groups 30-36 was measured at 38 weeks GA again. This allowed us to asses the long-term memory (LTM) of these fetuses, since this is reflected by the difference between HR-A and HR-C. However, this difference not only results from ‘memorizing’ the earlier habituation measurements at GA 30-36, but also from the normal GA-associated brain development during the 2-8 weeks between

both test sessions. To correct for this, the difference between HR-A and HR-C values (in % of HR-A) was decreased by the difference (also in % of HR-A) between each individual HR-A value and the median HR-A value (11.0) of a control group of 20 fetuses measured at 38 weeks GA. Consequently, individual long-term memory values were calculated according to the equation LTM = {100*[(HR-A minus HR-C)/(HR-A)]} minus {100*[(HR-A minus 11.0]/(HR-A)]}.

Cord sampling and fatty acid measurements

From the fetuses of groups 30-36, a piece of the umbilical cord was collected immediately after birth, rinsed with saline and stored at -80 °C until fatty acid analysis. It was decided to analyze only PLs of the artery walls, because this tissue represents the lowest essential fatty acid concentration available to more 'upstream' tissues in the fetal body. These fatty acid compositions were determined by capillary gas-liquid chromatography as described elsewhere [26,68] and expressed as relative values (% by wt of total identified PL-associated fatty acids). Fatty acid values < 0.05 % were considered too low for reliable detection and treated as missing. The selected fetal ePUFAs of interest were the major LCPUFAs for brain development, AA and DHA, their respective dietary precursors, the EFAs linoleic acid (18:2n-6, LA) and α-linolenic acid (18:3n-3, ALA) and three ePUFA status markers [5] (Osbond acid, 22:5n-6, ObA; Mead acid, 20:3n-9, MA; dihomo-Mead acid, 22:3n-9, DHMA).

Covariables

Parity [69], maternal smoking [70] and drinking during pregnancy [71], socio-economic status (SES) [72] and infant sex [73] were included in the multivariable-adjusted regression analyses as potential confounding factors. These characteristics were obtained via study questionnaires and medical records, as detailed before [67]. In the regression analyses two dummy variables for parity were used, one for parity = 1 and one for parity ≥ 2, with parity = 0 as reference category. Maternal smoking during pregnancy was categorized as 0 = non-smoking and 1 = 1-5 cigarettes per day. Maternal drinking during pregnancy was classified as 1 = 1 glass per week and 2 = 2-7 glasses per week, with 0 = no alcohol use as reference category, and infant sex as boy = 0 and girl = 1. Exact information on SES was not available. Therefore, parental SES was measured by proxy, using the variable 'highest educational level' [58]. For this variable, the education levels from both parents were compared and the highest education level (measured on an 8-point scale) was chosen as the value for the socio-economic status.

Statistical analyses

All data are presented as median (25^{th} - 75^{th} percentile), unless otherwise mentioned.

Associations between various fetal learning and memory outcome measures and the neonatal fatty acid concentrations of interest were analyzed with unadjusted and multivariable-adjusted regression analyses. In these analyses, fetal learning (HR-A), STM-1, STM-2 and LTM were the dependent variables and the relative proportions of the selected neonatal fatty acids LA, ALA, AA, DHA, ObA, MA, DHMA and MA+DHMA measured in PLs of the umbilical artery wall were the independent variables. Parity, SES (parental education), maternal smoking and drinking habits during pregnancy and infant sex were included as potential confounders.

At first, the selected ePUFA status markers were validated, based on our own fatty acid data, using Spearman's rank correlation test to check if it was appropriate to select these fatty acids as deficiency markers for the ePUFA status. For this test the markers (MA, DHMA, MA+DHMA and ObA) were correlated with LA, AA, DHA and the sums of the n-3 and n-6 fatty acids.

Habituation rates of the various groups were correlated to GA using Spearman's rank correlation test to check if habituation was GA-dependent. Since this was the not the case ($p > 0.050$), fetuses of groups 30-36 were combined to one group. However, as an extra check the variable 'group' was added to the list of potential confounders to correct for this factor, if appropriate.

Distributions of the dependent variables appeared skewed (Shapiro-Wilk test). Therefore, the distributions were optimized towards normal by means of transformations of the various datasets (natural log, square root, square or 1/square). Subsequently, obvious outliers (± 4 standard deviations (SD) outside the mean) were removed, after which the normality of the distributions was checked again. Since they still appeared not normal, the 4-SD outliers were inserted again and outliers were then removed if their values were more than 3 interquartile ranges (IQR) below or above the median. Although these procedures improved the distributions, they did not normalize them. Nonetheless, linear regression analyses were performed, but since none of the residuals were normally distributed, results could not be accepted. Therefore, all dependent variables were dichotomized (≥ median vs. < median) and logistic regression analyses were performed.

Unadjusted logistic regression analyses were carried out with the same subjects as included in the corresponding multivariable-adjusted regression analyses. Because of occasionally missing observations, this limited the number of cases for analysis. Therefore, to increase the number of available cases, irrelevant covariables were removed by stepwise backward multivariable-adjusted regression analyses, performed for each fatty acid-brain function combination. This procedure has been described in detail before [(74)]

and the successive steps were continued until all remaining covariables were either significant or were characterized as confounders. For each particular combination of fatty acid and fetal learning or memory criterion, these various steps were performed with the same dataset. However, since removal of the irrelevant covariables implied less missing values and, consequently, a larger number of cases available for analysis, the ultimate regression analyses were finally repeated with the maximum number of complete cases available for each combination of fatty acid and fetal brain function.

To check whether the relationships between dependent and independent variables were comparable for the added cases and the initial study population (a prerequisite for acceptance of this procedure), interaction analyses were performed as detailed before [74]. If the added cases were significantly different, the final model with the larger number of cases could not be accepted. Since these interaction analyses revealed no significant differences between initial and additional cases, all final backward models could be approved. Cook's distances were calculated to check for influential data points and data with Cook's distances > 1 were removed.

Subjects with incomplete information were nevertheless included. Therefore, not all data analyses were based on the same number of subjects. For both correlation studies, a p-value < 0.050 was considered significant. For all regression analyses, a p-value < 0.010 was required for significance, to correct for multiple testing, whereas a p-value < 0.050 was considered to indicate a (non-significant) trend. All statistical analyses were performed using the statistical package SPSS 11.5 for Windows (release 11.5, SPSS Inc., Chicago, Illinois).

Results

From the 80 participants included in groups 30-36, nine had to be excluded because of various pregnancy complications or because their fetuses did not react to the VAS at the initial habituation test of the first session (HR-A). Due to time restrictions, five of them could not be replaced, leaving 75 volunteers who completed the study. Three additional cases were excluded because cords could not be collected. Consequently, the data of 72 fetuses were left for analysis. In the control group (group 38), 2 fetuses were excluded because they did not react to the VAS at HR-A, remaining 18 fetuses instead of 20. All included neonates were in good health after birth, with a 5-minute Apgar score ≥ 8 and a birth weight > 10^{th} percentile, and no congenital anomalies were detected.

The relevant maternal and infant characteristics of groups 30-36 and the control group are listed in **table 1**. Results for fetal learning and memory are presented in **table 2**. The relative contents (% wt/wt) of the selected umbilical

artery wall PL fatty acids are reported in **table 3**. In most cases ALA levels were below the level of reliable detection and therefore ALA was left out of the statistical analyses. The unadjusted and multivariable-adjusted analyses were performed with the same number of complete cases (all (co)variables available). In general, increasing the power by including all available cases in the unadjusted regression analyses hardly affected the outcome of these analyses (results not shown).

Table 1. Maternal and infant characteristics

Characteristics	Group 30 - 36		Control group (group 38)	
	n	Median (25^{th} - 75^{th} percentile)	n	Median (25^{th} - 75^{th} percentile)
Maternal characteristics				
Age (years)	72	31 (28 - 34)	18	29.5 (27.5 - 32.3)
Educational level	71	6 (5 - 8)	18	6.0 (4.0 - 8.0)
Smoking during pregnancy (n) no/1-5 cigarettes per day	72	70/2	18	18/0
Alcohol use during pregnancy (n) no/1 glass per week/2-7 glasses per week	72	63/7/2	18	17/1/0
Parity (n) 0/1/2/3	72	39/21/10/2	18	9/7/2/0
Infant characteristics				
Birth weight (g)	72	3540 (3058 - 3945)	18	3630 (3320 - 3825)
Gestational age at delivery (weeks)	72	40.1 (39.3 - 40.7)	18	40.4 (39.9 - 41.0)
Sex (n) male / female	72	25/47	18	10/8
Apgar score after 5 minutes	71	10 (10 - 10)	18	10 (10 - 10)

Data are given as median (25^{th} - 75^{th} percentile), unless otherwise mentioned.

Table 2. Results for fetal learning and memory variables

Variables	n	Median (25^{th} - 75^{th} percentile)
Fetal learning	71	10.0 (5.0 - 16.0)
STM-1	65	76.2 (53.4 - 88.9)
STM-2	65	66.7 (22.9 - 90.9)
LTM	66	53.6 (0 - 102.5)

Fetal learning = non-transformed results of first habituation test outcome (HR-A); STM-1 and STM-2 = fetal short-term (10 minutes) memory, calculated as the difference in habituation rates between two successive habituation rates measured with a 10-minutes interval in respectively the first (HR-A and HR-B) and second session (HR-C and HR-D), and expressed in % of HR-A and HR-C, respectively; LTM = long-term memory, defined as the HR difference (%) between HR-A and HR-C, corrected for the normal GA-associated brain development.

Table 3. Relative contents (% wt/wt) of selected fatty acids isolated from arterial cord PLs (n = 72)

Fatty acids	Median (25^{th} - 75^{th} percentile)
LA	1.15 (0.98 - 1.28)
AA	13.7 (12.4 - 15.2)
DHA	5.81 (5.13 - 6.52)
ObA	3.19 (2.72 - 3.46)
MA	2.96 (2.39 - 3.68)
DHMA	1.57 (1.26 - 1.91)
MA+DHMA	4.54 (3.81 - 5.49)

PLs = phospholipids; LA = linoleic acid, 18:2n-6; AA = arachidonic acid, 20:4n-6; DHA = docosa-hexaenoic acid, 22:6n-3; ObA = Osbond acid, 22:5n-6; MA = Mead acid, 20:3n-9; DHMA = dihomo-Mead acid, 22:3n-9.

Validation of the ePUFA status markers

Using Spearman's rank correlation test, it was observed that the ePUFA status markers MA, DHMA and MA+DHMA were significantly negatively correlated with LA, AA, DHA and the sums of n-3 and n-6 fatty acids ($p < 0.001$; $.421 < r < .866$). For the status marker ObA significant negative correlations were observed with LA ($p = 0.029$; $r = .260$), DHA ($p = 0.047$; $r = .235$) and the sum of n-3 fatty acids ($p = 0.012$; $r = .294$). These results clearly demonstrate the suitability of MA, DHMA and MA+DHMA as general ePUFA status markers.

ObA, on the other hand, appears a more specific status marker of n-3 LCPUFAs in general, although the correlations were relatively weak.

Relationship between fetal learning (HR-A) and selected fatty acids

Neither in unadjusted, nor in fully adjusted or backward logistic regression analyses was fetal learning significantly associated with any of the fatty acids investigated, nor did they indicate a trend.

Relationship between short-term (10 minutes) memory and selected fatty acids

Unadjusted logistic regression analyses revealed trends between STM-1 and the fatty acids LA (n = 64; B = -2.067; p = 0.042; Odds Ratio (OR) = 0.127; 95 % confidence interval (CI) = (0.017;0.926); r^2 = 0.094), MA (n = 61; B = 0.714; p = 0.033; OR = 2.041; 95 % CI = (1.060;3.932); r^2 = 0.106) and MA+DHMA (n = 64; B = 0.430; p = 0.049; OR = 1.538; 95 % CI = (1.002;2.360; r^2 = 0.085). However, after adjustment for all covariables all these trends disappeared. After removal of irrelevant covariables by the stepwise backward procedure two positive trends were observed again for MA (n = 65; B = 0.716; p = 0.026; OR = 2.046; 95 % CI = (1.090;3.842); r^2 = 0.108) and MA+DHMA (n = 65; B = 0.452; p = 0.039; OR = 1.571; 95 % CI = (1.024;2.410); r^2 = 0.093). For these two backward analyses no covariables remained in the final model.

No other associations or trends between short-term (10 minutes) memory and the neonatal fatty acids of interest were found.

Relationship between long-term memory and selected fatty acids

In the unadjusted logistic regression analyses between LTM and ObA a positive trend was observed (n = 61; B = 1.148; p = 0.032; OR = 3.153; 95 % CI = (1.105;8.991); r^2 = 0.112). After full adjustment this positive association even became significant (n = 61; B = 2.740; p = 0.005; OR = 15.482; 95 % CI = (2.330;102.877); r^2 = 0.183). After the stepwise backward procedure, ‘maternal drinking during pregnancy’ and ‘group’ were left as confounders, and a positive trend remained (n = 63; B = 1.808; p = 0.012; OR = 6.097; 95 % CI = (1.489;24.967); r^2 = 0.137).

No other associations or trends between long-term memory and the neonatal fatty acids of interest were found.

Discussion and conclusions

The aim of this study was to investigate whether there are significant associations between fetal learning and memory, as assessed by fetal habituation measurements, and the fetal ePUFA status, reflected by the concentrations of AA, DHA, their dietary precursors and three ePUFA status markers, measured in cord artery wall PLs. We observed no distinct relations with AA or DHA, which are thought to be important LCPUFAs for brain development and function [37,38]. Also no significant associations or trends were observed between LA and fetal learning or memory. On the other hand, positive trends were observed between fetal STM-1 and levels of the ePUFA status markers MA and MA+DHMA and between fetal LTM and the n-3 LCPUFA status marker ObA. If causal, these relationships indicate that fetal short-term (10 minutes) memory measured before 38 weeks GA may be better, the lower the ePUFA status of the fetus, as reflected by higher MA and MA+DHMA levels. Likewise, fetal long-term memory would be better the lower the n-3 LCPUFA status, as indicated by higher ObA concentrations [75]. These interpretations are in striking contrast with current opinions, however.

Several human studies investigated the associations between maternal or neonatal LCPUFA concentrations measured during pregnancy or directly after delivery and children's brain development. In the majority of cases these studies addressed visual and cognitive development. Some of these studies observed positive associations, especially for DHA [76,77], whereas others found no significant relationships [78,79]. Also from a number of reviews it can be concluded that there is evidence for potential benefits of LCPUFAs on visual and cognitive development, but results are limited and often inconsistent [3,80-82]. It is difficult to compare these previous studies directly with our present one, since study designs and brain function measurements are so different. Furthermore, it must be kept in mind that the subjects of the present study were fetuses, whereas all other studies included infants in the age range from birth till a couple of years.

As far as we know, only a few studies used a brain function assessment procedure (the Fagan Test of Infant Intelligence [83]) more or less similar to the method we applied. In the habituation phase of this Fagan test, the investigator shows the infant two identical pictures of an infant's face, until habituation is reached. In the test phase, the original stimulus is then paired with a novel stimulus (picture of second face) and the investigator records the infant's looking direction and looking time at each stimulus. From these data the 'novelty preference' is calculated (the percentage of the total test time in the test phase that the infant spent looking at the novel stimulus). This test reflects the infant's ability to encode a stimulus into memory, to recognize that stimulus and to look preferentially at a novel stimulus. Oken and coworkers used this technique to assess associations between maternal fish and seafood intake

during the second trimester and infant cognition at 6 months of age. The results of this study showed that higher fish consumption of mothers during pregnancy was associated with better visual recognition memory of their infants at 6 months of age, especially after adjustment for maternal hair mercury levels [84]. Since a higher fish intake during pregnancy has been shown to be associated with a higher n-3 LCPUFA status of mothers and their neonates [85], this study suggests that the early availability of n-3 LCPUFAs may promote early brain development and function. Furthermore, also positive associations were found between cord plasma DHA concentrations and the Fagan test of novelty preference in a study of Jacobson et al. [86]. On the other hand, in several LCPUFA supplementation studies no significant effect of n-3 LCPUFA supplementation of babies or pregnant and lactating women on the Fagan test [87-89] were observed, although O'Conner found a positive influence for supplements containing both DHA and AA [90].

The Spearman rank correlation test outcome showed that the habituation rates were GA-independent and therefore fetuses of groups 30-36 were combined to one group. However, we added the variable 'group' as an extra check to the list of potential confounders to correct for this factor, if necessary. Indeed, in some multivariable analyses, 'group' appeared a confounder. Since habituation rates were GA-independent, this might indicate that other undefined factors besides gestational age are related with 'group' and influence the association between several forms of brain function and the selected fatty acids.

One of the selected ePUFA status markers, ObA, is thought to be synthesized when there is a functional shortage of DHA [14,15]. However, it needs to be realised that there is some evidence suggesting that ObA may not always be a useful biochemical measure of a low DHA status under all conditions [16]. On the other hand, we observed negative correlations ($p < 0.050$) for the relationships between the concentrations of ObA and those of DHA and the sum of the n-3 fatty acids. This demonstrates that it was appropriate to choose ObA as a status marker for the sum of n-3 fatty acids in particular, but it must be kept in mined that results were rather weak. For MA, DHMA and MA+DHMA levels strong negative correlations were observed with all ePUFAs. These latter results show that it was a correct decision to use these fatty acids as markers for the ePUFA status.

As mentioned before, in the present study only three trends were found for the associations between fetal brain functions and the early ePUFA availability. These trends imply negative associations between several fetal brain functions and the early ePUFA status, as reflected by higher concentrations of ObA, MA and MA+DHMA. Because of this small number of weak associations observed, our results might indicate that habituation-based brain functions are probably not related to the presence of the selected fatty acids, possibly because habituation is such a basic function, that it is optimal early in fetal development already. Indeed, these elementary forms of learning and memory are already

present in such primitive animals as worms and snails [91,92], which have a relatively simple neural network.

In conclusion, since only a few trends were observed for the associations between habituation-related fetal brain functions and the early ePUFA status, we concluded that physiological differences in the availability of these fatty acids may probably not determine the differences in these primitive brain functions during the third trimester of fetal development.

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Chapter 4

Associations between neonatal birth dimensions and maternal essential and *trans* fatty acid contents during pregnancy and at delivery

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Abstract

Since birth dimensions have prognostic potential for later development and health, possible associations between neonatal birth dimensions and selected maternal plasma fatty acid contents were investigated, using data from 782 mother-infant pairs of the Maastricht Essential Fatty Acid Birth cohort. Unadjusted an multivariable-adjusted regression analyses were applied to study the associations between birth weight, birth length or head circumference and the relative contents of docosahexaenoic acid (DHA), arachidonic acid (AA), dihomo-γ-linolenic acid (DGLA) and 18:1*trans* (18:1*t*) in maternal plasma phospholipids sampled during early, middle and late pregnancies, and at delivery. Where appropriate, corrections were made for relevant covariables**.** Significant *positive* associations were observed between maternal DHA contents (especially early in pregnancy) and birth weight (B = 52.10 g, 95 % CI = 20.40 - 83.80) and head circumference (B = 0.223 cm, 95 % CI = 0.074 - 0.372). AA contents at late pregnancy were *negatively* associated with birth weight (B = -44.25 g, 95 % CI = -68.33 - -20.16) and birth length (B = -0.200 cm, 95 % CI = -0.335 - -0.065). Significant *negative* associations were also observed for AA contents at delivery and birth weight (B = -27.08 g, 95 % CI = -47.11 - -7.056) and birth length (B = -0.207 cm, 95 % CI = -0.330 - -0.084). Maternal DGLA contents at delivery were also significantly *negatively* associated with neonatal birth weight (B = -85·76 g, 95 % CI = -130.9 - -40.61) and birth length (B = -0.413 cm, 95 % CI = -0.680 - -0.146). No significant associations were observed for maternal 18:1*t* contents. We conclude that during early pregnancy, maternal DHA content may programme fetal growth in a positive way. Maternal AA and DGLA in late pregnancy might be involved in fetal growth limitation.

Introduction

Increasing evidence suggests that birth dimensions have prognostic values for later development and health. Indeed, birth weight and birth length have been shown to be associated with later cardiovascular risk [93], whereas head circumference at birth seems a significant predictor of later intelligence [94]. Although the causality of these associations has not yet been demonstrated, fetal growth should be optimized.

The essential long-chain polyunsaturated fatty acids (LCPUFAs), docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6), are thought to be of critical importance for brain development and fetal growth, respectively [2,3]. Therefore, the availability of DHA and AA to the fetus needs to be adequate. To obtain these fatty acids, fetuses depend on their mothers, as is indicated by the positive correlation between the maternal and neonatal LCPUFA status at birth [6-8]. Mothers receive the LCPUFAs mainly from their diet or synthesize them from their respective precursors [95].

Dietary *trans* fatty acids are unsaturated fatty acids with at least one double bond in the *trans* configuration. They mainly result from industrial hydrogenation of edible oils and cannot be synthesized by humans [18]. As is the case for LCPUFAs, maternal and neonatal *trans* fatty acids are positively correlated [20,21], indicating that the neonatal *trans* status is also determined by the maternal *trans* intake. Dietary *trans* unsaturated fatty acids have been shown to inhibit the conversion of parent essential fatty acids (EFAs) into their LCPUFAs, especially when the EFA contents are low [22,23]. They may also impair placental LCPUFA transfer [24]. Thus, *trans* fatty acids may lower the fetal LCPUFA status and thereby they could compromise fetal development.

Recently, the term birth weight appeared to be positively associated with most maternal n-3 LCPUFA proportions early in pregnancy [96]. The relationship with maternal AA contents was found to be negative. Interestingly, maternal contents of the AA precursor dihomo-γ-linolenic acid (DGLA, 20:3n-6) is positively related to the term birth weight. The birth-weight relationship with elaidic acid (the main dietary *trans* fatty acid, 18:1n-9*trans*,) was negative in unadjusted analyses, but this association lost significance after adjustment for covariables.

The aim of the present study was to extend and confirm these findings with three birth outcome measures (birth weight, birth length and head circumference at birth) and maternal plasma fatty acid proportions from four different sampling times (about 16, 22 and 32 weeks of pregnancy, and immediately upon delivery), available from a different birth cohort.

Subjects and methods

General design of the study

On the basis of distinct inclusion criteria, relevant data (dependent variables, independent variables and covariables) of eligible mothers and their infants were extracted from the database of the Maastricht Essential Fatty Acid Birth (MEFAB) cohort. This database contains the data of pregnant women and their newborns who participated in several observational studies, conducted in our institute between 1990 and 1997 [1,7]. In short, three antenatal clinics located in The Netherlands participated and recruited pregnant women at their first antenatal medical visit. Selection criteria for inclusion were a gestational age < 16 weeks at study entry, singleton pregnancy, Caucasian race, diastolic blood pressure < 90 mm Hg and the absence of any metabolic, cardiovascular, neurological or renal disorder at the time of recruitment. Approval for these studies was obtained from the Medical Ethics Committee of the University Hospital Maastricht and the University of Maastricht, and all participating women gave their written informed consent. Unadjusted and multivariable-adjusted linear regression analyses were conducted to study the associations between relative DHA, AA, DGLA and 18:1*trans* (18:1*t*) contents in phospholipids (PLs) of maternal plasma, collected at approximately 16, 22 and 32 weeks of pregnancy, and directly after delivery (independent variables), and the birth outcome measures birth weight, birth length or head circumference (dependent variables). In multivariable-adjusted analyses, these relationships were corrected for relevant covariables.

Inclusion of participants

For the present study, clinical data of 1238 mothers and their infants were available in the MEFAB database. The mother-infant pairs were excluded if infants were born preterm (gestational age < 37 weeks, n = 90), mothers had diabetes (n = 35) or developed pregnancy-induced hypertension (n = 96), mothers had reported specific health problems in the past (e.g. diabetes mellitus, hypertension and heart, kidneys, liver, gall bladder or thyroid gland disorders, n = 128), one or both parents were non-Caucasians (n = 40) or values for any of the afore-mentioned exclusion criteria were missing (n = 73). The mother-infant pairs were also excluded if fatty acid analyses were not reported (n = 148) or values were missing for birth weight, birth length and head circumference (n = 3). After exclusion, the data of 782 mother-infant pairs were left for analysis.

Birth dimensions and fatty acid profiles

Local hospital staff members recorded birth weight, birth length and head circumference on standardized datasheets. Maternal venous blood samples were collected in EDTA tubes at about 16, 22 and 32 weeks of pregnancy, and immediately after delivery. The plasma was separated from the blood cells by centrifugation (2000 g, 4 ºC, 15 min) and stored under nitrogen at -80 ºC until analysis. The fatty acid composition of plasma PLs was determined by gas-liquid chromatography, as described previously [26]. The separation of the various 18:1*t* isomers was incomplete; therefore, they are reported together as 18:1*t*. The fatty acids are expressed as relative contents (percentage by weight of the total amount of identified fatty acids, % wt/wt).

Covariables

Maternal age [97], height and body mass index [BMI, weight (kg)/height (m^2)] at study entry [98], parity [99], smoking and drinking during pregnancy [100], weight increase during pregnancy [101], socio-economic status (income) [102], gestational age [98] and infant sex [103] were included in the multivariable-adjusted analyses as potential confounding factors. These data were retrieved from the original study questionnaires and medical records. In the multivariable-adjusted regression analyses, two dummy variables for parity were used, one for parity = 1 and the other for parity ≥ 2, with parity = 0 as reference category. Smoking and drinking during pregnancy were both categorized as 0 = no and 1 = yes and infant sex as boy = 0 and girl = 1. Exact information on socio-economic status (SES) was not available. Therefore, parental SES was measured by proxy, using 'income' as an SES indicator, based on the parental postal code at the time of delivery (Geomarktprofiel; Wegener DM, Nieuwegein, The Netherlands). This information was classified into five groups ranging from 1 (twice or more modal income) to 5 (minimum income); SES values in the categories unknown (0) and diverse (6) were omitted and thus reported as missing values. Gestational age at birth was calculated from the self-reported first day of the last menstrual period. If the last menstrual period was uncertain, gestational age was based on early ultrasound measurements.

Statistical analyses

Associations between various birth outcome measures and the maternal fatty acid contents of interest were analyzed with unadjusted and multivariable-adjusted linear regression analyses. In these analyses, birth weight, birth length and head circumference were the dependent variables and the relative proportions of the maternal fatty acids DHA, AA, DGLA and 18:1*t* measured in plasma PLs were the independent variables. In the multivariable-adjusted

models, the four fatty acids were included simultaneously to allow for their usual metabolic interactions [2,95]. Furthermore, since fatty acids are reported in relative contents, any change in the proportion of one fatty acid will result in a change in the proportions of the other fatty acids included in the analysis. Maternal age, height, BMI at study entry, parity, weight increase during pregnancy, socio-economic status (income), smoking and drinking habits during pregnancy, gestational age and infant sex were included as potential confounders. Before starting the analyses, outliers (± 4 SD outside the mean) of the dependent variables were removed and the normality of their distributions was checked and confirmed by histograms.

Unadjusted regression analyses were performed with the same subjects as included in the corresponding multivariable-adjusted regression analyses. These twelve regression analyses (three dependent variables and fatty acid contents at four time points) were performed in subjects with complete datasets for all (co)variables included in each of the respective regression models. Because of occasionally missing observations, the number of cases for analysis was limited. Ultimately, to remove irrelevant covariables (and thereby increase the number of available cases, see later), stepwise-backward multivariable-adjusted regression analyses were performed for each of the twelve birth outcome-fatty acid combinations. Starting with the full multivariable-adjusted model, the covariable with the highest p-value was removed. If this removal resulted in a change in the B-value of 10 % or more for at least one of the four fatty acids included (DHA, AA, DGLA and 18:1*t*), *and* if this change amounted to 20 % or more of the standard error of this B-value, then this variable was considered a confounder and retained in the regression model, even if it was not significant ($p \geq 0.050$) [104]. If the removal of the covariable resulted in a smaller change in the B-values for all the four fatty acids included and/or of their standard errors, then its removal was permanent. Subsequently, this procedure was repeated with the covariable with the next largest p-value and so on, until the remaining covariables were either significant (in which case they are called 'predictors') or characterized as confounders. For each particular birth outcome-fatty acid combination, these various steps were performed with the same dataset. However, since the removal of the irrelevant covariables implied less missing values and, consequently, a larger number of cases available for analysis, the ultimate regression analyses were finally repeated with the maximum number of complete cases available for that birth outcome-fatty acid combination.

To check whether the relationships between dependent and independent variables are comparable for the added cases and the initial study population (a prerequisite for acceptance of this procedure), interaction analyses were performed. To this end, two new variables were introduced into the final regression model. The first variable, named A, was coded '0' for cases from the initial (full) model and '1' for the cases that were added after the deletion of the

irrelevant covariables. The second variable was the interaction term, calculated as 'the respective independent fatty acid variable * variable A'. The significance of this interaction term ($p < 0.010$, to correct for multiple testing) implies that the regression models are fundamentally different for both sets of cases. In that case, the final model with the larger number of cases could not be accepted. Since these interaction analyses revealed no significant differences between initial and additional cases, all the final backward models could be approved. To check possible influential cases in the regressions, all data points were checked by calculating their Cook's distance and removed if this value was ≥ 1. Such influential data points were not observed, however.

For the regression analyses, p-values < 0.010 were considered statistically significant, to correct for multiple testing. A p-value < 0.050 was considered to indicate a (non-significant) trend. The values are reported as median (25^{th} - 75^{th} percentile), unless specified otherwise. All statistical analyses were performed using SPSS 11.5 for Windows (SPSS Inc., Chicago, IL, USA).

Results

A total of 782 mother-infant pairs enrolled in the present study. Their relevant characteristics are listed in **table 1**. The relative contents (% wt/wt) of the four maternal plasma PL fatty acids of interest are reported in **table 2**. In **tables 3-5**, results of the unadjusted, multivariable-adjusted and final backward regression analyses are shown.

Relationship between maternal 18:1t contents and birth dimensions

None of the associations between relative maternal 18:1*t* contents and birth weight, birth length or head circumference reached statistical significance or indicated a trend (results not shown). In addition, the backward regression analyses demonstrated that for none of the twelve birth outcome-fatty acid combinations, 18:1*t* was either a predictor or a confounder. Since a considerable number of 18:1*t* values were missing from the database, which lowered the number of cases and, consequently, the power of the regression, it was decided to remove 18:1*t* from the dataset and to restrict further analyses to the combination of the three LCPUFAs DHA, AA and DGLA. This decision appeared justified after the last check, demonstrating that also in the final backward analyses with the three LCPUFAs included, 18:1*t* was neither a confounder nor a predictor (data not shown).

Table 1. Subject characteristics

Maternal characteristics	n	
Age (years)	782	29.0 (26.2 - 31.7)
Height (cm)	740	167 (162 - 170)
BMI at study entry (kg/m^2)	709	23.0 (21.2 - 25.3)
Parity (n) 0 / 1 / ≥ 2	782	574 / 173 / 35
Weight increase during pregnancy (kg)	736	11.7 (9.2 - 14.3)
Socio-economic status (income class)[a]	559	3 (2 - 3)
Smoking during pregnancy (n) no / yes	778	570 / 208
Alcohol during pregnancy (n) no / yes	779	761 / 18
Infant characteristics		
Gestational age (weeks)	782	40.1 (39.3 - 41.0)
Sex (n) male / female	782	421 / 361
Birth weight (g)	780	3331 (448)
Birth length (cm)	661	50.1 (2.2)
Head circumference (cm)	580	34.2 (1.6)

Birth weight, birth length and head circumference were normally distributed and are therefore expressed as mean ± SD. The distributions of the other characteristics were not checked for normality and are therefore given as median (25th - 75th percentile). [a] Ranges from minimum (5) to ≥ 2 x modal (1).

Relationship between maternal DHA contents and birth dimensions

Unadjusted regression analyses revealed significant positive relationships between maternal DHA contents at week 16 of pregnancy and birth weight and head circumference (**Table 3**). After entering all covariables, these associations remained significant. After the removal of irrelevant covariables by the stepwise-backward procedure, the final models explained 37.5 and 20.0 % of the variability in birth weight and head circumference, respectively. The contributions of DHA stayed significant, explaining 1.0 and 1.4 % of these variabilities, respectively.

For birth length, unadjusted regression analyses revealed a significant positive association with maternal DHA, measured at 32 weeks pregnancy. This association just lost significance after adjustment for relevant covariables. The relationships between birth length and DHA proportions measured at 16 and 22 weeks were of the same order of magnitude, but they only showed non-

Table 2. Relative contents (% wt/wt) of selected fatty acids in maternal plasma PLs collected at several pregnancy durations (weeks) and at delivery

Fatty acids	n	16 weeks	n	22 weeks	n	32 weeks	n	Delivery
DHA	749	3.88 (3.34 - 4.49)	697	3.98 (3.46 - 4.51)	722	3.84 (3.39 - 4.44)	676	3.75 (3.31 - 4.24)
AA	749	9.59 (8.68 - 10.53)	697	8.60 (7.75 - 9.51)	722	8.14 (7.35 - 8.94)	676	8.47 (7.60 - 9.43)
DGLA	749	3.06 (2.67 - 3.54)	697	3.36 (2.98 - 3.74)	722	3.34 (2.96 - 3.76)	676	3.40 (3.05 - 3.82)
18:1*t*	574	0.45 (0.33 - 0.59)	534	0.44 (0.32 - 0.58)	553	0.42 (0.31 - 0.54)	516	0.37 (0.27 - 0.49)

The relative fatty acid results are expressed as median (25^{th} - 75^{th} percentile). PLs = phospholipids; AA = arachidonic acid; DGLA = dihomo-γ-linolenic acid; DHA = docosahexaenoic acid; 18:1*t* = 18:1*trans* isomers.

Table 3. Unadjusted and multivariable-adjusted (backward) regression analyses of the relationships between birth outcome variables and docosahexaenoic acid contents in PLs of maternal plasma, collected at different times during pregnancy and at delivery

Pregnancy duration (weeks)	Birth outcome	Unadjusted model (no covariables included)				Multivariable-adjusted model (all covariables included)[a,b]				Final multivariable-adjusted backward model (only relevant covariables included)[a,c]						
		n	R^2	B	p	R^2	B	r^2	p	n	R^2	B	r^2	p	95 % CI (B)	
															Low	High
16	BW[d]	473	0.018	64.12	*0.003*	0.357	61.97	0.015	*0.001*	665	0.375	52.10	0.010	*0.001*	20.40	83.80
	BL[d]	410	0.016	0.313	0.011	0.279	0.282	0.011	0.014	569	0.287	0.210	0.006	0.025	0.026	0.393
	HC[e]	369	0.022	0.255	*0.005*	0.181	0.275	0.022	*0.002*	510	0.200	0.223	0.014	*0.003*	0.074	0.372
22	BW[d]	441	0.001	17.09	0.462	0.337	23.75	0.002	0.263	623	0.362	31.18	0.003	0.085	-4.301	66.67
	BL[#]	384	0.011	0.277	0.040	0.275	0.295	0.010	0.021	536	0.283	0.270	0.009	0.010	0.065	0.476
	HC[g]	343	0.005	0.133	0.188	0.155	0.102	0.003	0.322	472	0.165	0.142	0.005	0.107	-0.031	0.314
32	BW[d]	457	0.003	28.41	0.272	0.355	32.01	0.003	0.164	644	0.368	33.08	0.003	0.094	-5.699	71.86
	BL[h]	396	0.017	0.379	*0.008*	0.286	0.329	0.011	0.015	551	0.285	0.276	0.008	0.012	0.060	0.491
	HC[e]	353	0.006	0.162	0.134	0.162	0.181	0.007	0.100	489	0.177	0.192	0.007	0.039	0.010	0.373
Delivery	BW[d]	467	0.002	-22.45	0.362	0.342	-12.63	0.000	0.578	608	0.367	3.423	0.000	0.861	-34.95	41.80
	BL[h]	407	0.001	0.069	0.629	0.269	0.131	0.002	0.346	526	0.287	0.157	0.003	0.171	-0.068	0.382
	HC[e]	363	0.000	0.042	0.690	0.148	0.044	0.000	0.688	467	0.187	0.191	0.007	0.050	0.000	0.382

BW = birth weight; BL = birth length; HC = head circumference; PLs = phospholipids. R^2 = coefficient of determination of total model; r^2 = square of the semi-partial correlation coefficient of fatty acid concerned; B = regression coefficient of fatty acid of interest; p = p-value of fatty acid concerned; CI = confidence interval. [a] The total model p-values of the (final) multivariable-adjusted analyses were all < 0.000. [b] Same cases as included in unadjusted model. [c] For all models the following covariables appeared relevant: infant sex, gestational age and maternal height; Additional relevant covariables: [d] parity, weight increase during pregnancy, body mass index (BMI) at study entry, smoking and drinking during pregnancy; [e] parity and body mass index (BMI) at study entry; [#] parity, smoking during pregnancy, body mass index (BMI) at study entry and weight increase during pregnancy; [g] parity and weight increase during pregnancy; [h] weight increase during pregnancy, body mass index (BMI) at study entry, smoking and drinking during pregnancy. *Italic numbers* refer to a significant relationship ($p < 0.010$), $0.010 \leq p < 0.050$ refer to a non-significant trend.

significant positive trends in unadjusted analyses as well as after adjustment for relevant covariables.

None of the birth outcome variables were significantly associated or showed trends with DHA values measured at delivery.

Relationship between maternal AA contents and birth dimensions

No significant associations were observed in unadjusted analyses between AA contents in maternal plasma PLs and birth outcome variables (**Table 4**). After adjustment for covariables, associations with neonatal head circumference hardly changed and remained insignificant. The adjustment *did* enhance most associations of birth weight and birth length with maternal AA proportions at 16 and 22 weeks of pregnancy, but only non-significant negative trends became apparent between maternal AA contents at week 16 and 22 of pregnancy and birth weight and between AA contents at 22 weeks of pregnancy and birth length. When related to maternal AA proportions in late pregnancy (week 32) and at delivery, however, *significant* negative relationships were observed. The significant final multivariable-adjusted backward models (with only relevant covariables included) explained, respectively, up to 36.8 and 28.7 % of the variability in birth weight and birth length, up to 1.5 % of which was due to the contribution of AA.

Relationship between maternal DGLA contents and birth dimensions

Unadjusted regression analyses did not demonstrate any significant relationship between maternal DGLA contents and birth outcome variables (**Table 5**). However, after adjustment for only the relevant covariables, a negative trend was found for the relationship between maternal DGLA proportions at 32 weeks pregnancy and birth weight. After full adjustment, the maternal DGLA content at delivery was significantly and negatively associated with neonatal birth weight. The complete model explained about one-third (34.2 %) of the variability in birth weight, 1.4 % of which was contributed by DGLA. After the removal of the irrelevant covariables by the stepwise-backward procedure, the final model explained 36.7 % of the variability in birth weight and the contribution of DGLA (1.5 %) remained significant. For the association between maternal DGLA proportions at delivery and neonatal birth length, comparable results were obtained. The full multiple regression model explained 26.9 % of the variability in birth length with an almost significant contribution of 0.8 % from DGLA. This contribution increased to 1.3 % and became significant after the removal of irrelevant covariables by the backward regression analysis.

Other associations between birth outcome variables and maternal DGLA contents were not significant and no trends were found either.

Table 4. Unadjusted and multivariable-adjusted (backward) regression analyses of the relationships between birth outcome variables and arachidonic acid contents in PLs of maternal plasma, collected at different times during pregnancy and at delivery

Pregnancy duration (weeks)	Birth outcome	Unadjusted model (no covariables included)				Multivariable-adjusted model (all covariables included)[a,b]				Final multivariable-adjusted backward model (only relevant covariables included)[a,c]						
		n	R^2	B	p	R^2	B	r^2	p	n	R^2	B	r^2	p	95 % CI (B)	
															Low	High
16	BW[d]	473	0.000	1.468	0.912	0.357	-22.14	0.005	0.060	665	0.375	-20.75	0.004	0.037	-40.22	-1.274
	BL[d]	410	0.000	0.008	0.914	0.279	-0.103	0.004	0.141	569	0.287	-0.090	0.003	0.117	-0.203	0.023
	HC[e]	369	0.000	0.014	0.791	0.181	-0.067	0.004	0.214	510	0.200	-0.078	0.004	0.094	-0.169	0.013
22	BW[d]	441	0.001	-7.785	0.604	0.337	-31.84	0.008	0.024	623	0.362	-25.90	0.005	0.030	-49.32	-2.478
	BL[#]	384	0.001	-0.051	0.554	0.275	-0.207	0.012	0.014	536	0.283	-0.174	0.009	0.012	-0.310	-0.038
	HC[g]	343	0.006	0.090	0.167	0.155	0.031	0.001	0.650	472	0.165	0.020	0.000	0.723	-0.091	0.131
32	BW[d]	457	0.009	-32.85	0.042	0.355	-49.22	0.017	*0.001*	644	0.368	-44.25	0.013	*0.000*	-68.33	-20.16
	BL[h]	396	0.004	-0.105	0.232	0.286	-0.220	0.013	*0.009*	551	0.285	-0.200	0.011	*0.004*	-0.335	-0.065
	HC[e]	353	0.000	0.020	0.772	0.162	-0.022	0.000	0.762	489	0.177	-0.072	0.003	0.231	-0.190	0.046
Delivery	BW[d]	467	0.008	-25.05	0.052	0.342	-38.91	0.016	*0.001*	608	0.367	-27.08	0.008	*0.008*	-47.11	-7.056
	BL[h]	407	0.004	-0.098	0.186	0.269	-0.192	0.013	*0.008*	526	0.287	-0.207	0.015	*0.001*	-0.330	-0.084
	HC[e]	363	0.001	0.029	0.611	0.148	0.008	0.000	0.893	467	0.187	-0.033	0.001	0.520	-0.132	0.067

For explanations of symbols, see **table 3**.

Table 5. Unadjusted and multivariable-adjusted (backward) regression analyses of the relationships between birth outcome variables and dihomo-γ-linolenic acid contents in PLs of maternal plasma, collected at different times during pregnancy and at delivery

Pregnancy duration (weeks)	Birth outcome	Unadjusted model (no covariables included)				Multivariable-adjusted model (all covariables included)[a,b]				Final multivariable-adjusted backward model (only relevant covariables included)[a,c]						
		n	R^2	B	p	R^2	B	r^2	p	n	R^2	B	r^2	p	95 % CI (B)	
															Low	High
16	BW[d]	473	0.002	24.75	0.400	0.357	8.829	0.000	0.732	665	0.375	-5.410	0.000	0.811	-49.78	38.96
	BL[d]	410	0.000	0.026	0.875	0.279	-0.035	0.000	0.819	569	0.287	-0.041	0.000	0.750	-0.294	0.212
	HC[e]	369	0.000	0.028	0.825	0.181	0.106	0.002	0.395	510	0.200	0.126	0.002	0.243	-0.086	0.337
22	BW[d]	441	0.003	37.64	0.237	0.337	-3.640	0.000	0.899	623	0.362	-16.20	0.000	0.520	-65.64	33.25
	BL[#]	384	0.001	0.121	0.496	0.275	0.029	0.000	0.862	536	0.283	-0.055	0.000	0.703	-0.340	0.229
	HC[g]	343	0.006	0.202	0.143	0.155	0.257	0.008	0.071	472	0.165	0.101	0.001	0.393	-0.132	0.335
32	BW[d]	457	0.000	10.31	0.748	0.335	-38.30	0.003	0.190	644	0.368	-54.55	0.005	0.029	-103.6	-5.471
	BL[h]	396	0.000	-0.053	0.764	0.286	-0.161	0.002	0.345	551	0.285	-0.269	0.005	0.053	-0.542	0.004
	HC[e]	353	0.002	0.100	0.462	0.162	0.177	0.004	0.215	489	0.177	0.005	0.000	0.969	-0.228	0.237
Delivery	BW[d]	467	0.002	-28.96	0.337	0.342	-84.00	0.014	*0.002*	608	0.367	-85.76	0.015	*0.000*	-130.9	-40.61
	BL[h]	407	0.003	-0.187	0.271	0.269	-0.340	0.008	0.033	526	0.287	-0.413	0.013	*0.003*	-0.680	-0.146
	HC[e]	363	0.001	-0.083	0.518	0.148	-0.050	0.000	0.698	467	0.187	-0.136	0.003	0.236	-0.361	0.089

For explanations of symbols, see **table 3**.

Discussion

In the present study, with data of mother-infant pairs present in the MEFAB database, we observed that the plasma PL DHA contents of mothers, especially when measured early in pregnancy, were significantly and *positively* related to birth weight and head circumference of their neonates, whereas maternal AA and DGLA proportions in late pregnancy were *negatively* related to birth weight and birth length. No significant associations were observed for maternal contents of 18:1*t*, a group of industrial *trans* unsaturated fatty acid present in the diet.

In the literature, most observational studies relating neonatal birth outcome to maternal n-3 fatty acid intake inferred from the intake of fish and marine mammals assessed using food frequency questionnaires. A few of these studies reported positive associations between n-3 fatty acids and birth dimensions [40,105,106], whereas other studies found no [41,107] or even negative associations [42]. No significant associations were reported between birth weight or birth length and maternal DHA contents measured in plasma lipid fatty acids at 35 weeks of pregnancy [8] or the fatty acid ratio (EPA + DPA + DHA)/AA in erythrocytes sampled at 30 weeks of pregnancy and used as a biochemical marker of marine n-3 fatty acid intake [107].

The reasons for these inconsistent results probably include the use of different methods to estimate the n-3 LCPUFA status of the women, as well as incomplete adjustment for covariables [8,40-42,105]. Furthermore, most observational studies were restricted to relationships with maternal fatty acids in late pregnancy, whereas we observed significant associations with maternal DHA contents early in pregnancy only. This finding suggests a fetal growth programming potential of maternal DHA, especially, early in pregnancy, which (if confirmed) could imply that DHA supplementation after this period may not significantly affect birth dimensions anymore. Indeed, as initially observed by Olsen et al. [108] and recently confirmed by a meta-analysis containing five additional randomized controlled trials, no significant effects on birth weight or birth length are observed when n-3 LCPUFAs are supplemented from 15 to 30 weeks of pregnancy onwards [109].

The present results agree with those of van Eijsden et al. who observed a significant positive correlation between DHA proportions measured in maternal plasma PLs at 13 weeks of pregnancy and birth weight. However, this association lost significance after adjustment for covariables [96]. It should be mentioned that, when compared with our volunteers, their (multi-ethnic) population had relatively high DHA values, and the association they observed was mainly present at the lower range of the DHA distribution.

Previous observational studies suggested that AA has growth-promoting effects early in life both in preterm [110,111] and term neonates [8,112,113]. By contrast, we observed negative associations between AA contents in maternal

plasma PLs and neonatal birth weight and length. Although present throughout pregnancy, these associations were only significant at late pregnancy and directly after delivery, which suggests involvement of AA in fetal growth limitation. These results confirm earlier observations of van Eijsden and co-workers that higher AA proportions in plasma PLs of women at about 13 weeks of pregnancy are significantly related to lower birth weights of their neonates [96]. At 16 weeks of pregnancy, the negative association we observed in the present study for birth weight was not quite significant ($p = 0.037$), which may have been due to the smaller study population ($n = 665$), compared with the study of van Eijsden ($n = 3706$).

In general, maternal plasma contents of DGLA, the precursor of AA, were negatively associated with birth weight and birth length after correction for relevant covariables. Although not significant for fatty acid proportions early in pregnancy, the associations became significant as pregnancy progressed. From the literature, hardly anything is known about the possible association between maternal DGLA contents and birth outcome. In their (uncorrected) study, Elias and Innis did not observe a significant relationship with DGLA contents measured in maternal plasma PLs, triacylglycerol or cholesteryl esters at 35 weeks of pregnancy for birth weight and birth length [8]. It should be mentioned that in the present study, significant results for DGLA were observed only after correction for relevant covariables. van Eijsden and co-workers found a significant *positive* association between maternal DGLA proportions, measured early in pregnancy, and birth weight in unadjusted and multivariable-adjusted analyses ($p < 0.010$) [96]. Although we tested several possible scenario's, we were unable to reconcile these contrasting results.

In agreement with the inhibitory effect of *trans* unsaturated fatty acids on the endogenous LCPUFA synthesis from their EFA precursors and on their placental transfer [22-24], 18:1*t* contents in maternal plasma PLs were found significantly and negatively associated with maternal DHA and AA proportions measured during pregnancy and directly after delivery (data not shown). Despite the rather strong relationships ($p \leq 0.011$) and the significant associations we observed between maternal LCPUFA contents and birth dimensions, associations between maternal 18:1*t* proportions and the various birth outcome variables were not significant. This might be partly explained by the rather low 18:1*t* contents in the plasma PLs of the present study population (**Table 2**), resulting in a narrow exposure range, which usually impedes the detection of any association. As in the (unadjusted) analyses of Elias and Innis, the *trans* fatty acid proportions were also not significantly related to infant birth weight and birth length [8], it seems that any potential effect of industrial *trans* unsaturated fatty acids is either small or non-existing.

One of the strengths of the present study is that plasma for fatty acid content measurement was collected at several time points during pregnancy and directly after birth. This allowed us to investigate the associations of these

fatty acids with birth dimensions from early pregnancy onwards until and including delivery. Second, the database of this cohort contains a relatively large number of maternal and neonatal covariables. However, as with all observational studies, residual confounding cannot be ruled out and it is not possible to decide whether or not the associations observed are causal.

Even though significant correlations existed between DHA, AA, DGLA and 18:1*t*, we regarded it justified to include them in the multivariable-adjusted analyses together, because the correlation coefficients between these fatty acids were ≤ 0.4. In addition, the multicollinearity checks revealed a tolerance value of > 0.1 and a variance inflation factor of < 10, which allowed us to make this decision [114]. By including the fatty acids simultaneously, it was first possible to take into consideration their metabolic interactions [2,95]. Second, since fatty acid contents are reported in proportions, any change in the proportion of one fatty acid will result in a change in the relative proportions of the other fatty acids included in the analysis.

To check whether confounding was present and in what direction it worked, the same subjects were used in the unadjusted as well as the multivariable-adjusted regression analyses. When unadjusted regression analyses were performed with the maximum number of cases available, the associations were considerably more significant than those reported in **tables 3-5** (data not shown).

The present results support earlier suggestions [96] that differences in maternal LCPUFA contents (irrespective of their causes) can have a significant impact on neonatal birth dimensions. Moreover, the present results can be taken to indicate that maternal DHA contents may programme fetal growth in a positive way, whereas maternal AA and DGLA later in pregnancy might be involved in fetal growth limitation. If these associations prove to be causal, they imply that birth dimensions can be optimized by the adaptation of the maternal LCPUFA intake during pregnancy, because circulating proportions of LCPUFA depend, at least partly, on LCPUFA intake. Thus, intervention studies clearly demonstrated that plasma contents of DHA, AA and DGLA, as well as their proportions, can be modified by changing the consumption of these LCPUFAs or their precursors [95,115-117], although non-dietary factors can also play a modulating role [118-120]. Therefore, randomized clinical trials are needed to test the causality of the associations observed and to find the ideal maternal LCPUFA status for optimum fetal growth and infant development. As it is known that maternal and neonatal essential fatty acid contents are positively related with each other [6,7,26], such intervention studies would be especially appropriate if it can be confirmed that associations with birth dimensions, as observed in the present study for maternal LCPUFA contents during pregnancy, also exist for neonatal LCPUFA contents at birth. These studies are now underway.

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Chapter 5

Associations between term birth dimensions and prenatal exposure to essential and *trans* fatty acids

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Under revision

Abstract

Certain essential long-chain polyunsaturated fatty acids (LCPUFAs) are considered important for fetal growth and brain development, whereas industrial *trans* fatty acids (mainly 18:1*trans*) have been associated with negative effects. The aim of this study was to investigate associations between term birth dimensions and prenatal exposure to some of these fatty acids, reflected by neonatal fatty acid concentrations at birth. Data of up to 700 infant-mother pairs from the Maastricht Essential Fatty Acid Birth cohort were used for the present study. Unadjusted and multivariable-adjusted linear regression analyses were performed to investigate associations between birth weight, birth length or head circumference and relative concentrations of docosahexaenoic acid (DHA), arachidonic acid (AA), dihomo-γ-linolenic acid (DGLA) and *trans*-octadecenoic acids (18:1*t*) measured in phospholipids of the walls of umbilical arteries and veins, and in umbilical cord plasma and erythrocytes. After optimal adjustment, a significant negative association was observed between birth weight and umbilical plasma DHA concentrations. Negative associations were also found for AA concentrations measured in umbilical plasma and in arterial and venous vessel walls. Birth length was negatively related to arterial vessel wall AA concentrations only. A significant negative association was observed for the relationship between 18:1*t* in cord erythrocytes and birth weight. For DGLA no significant associations were observed. Results seem to preclude a role of DHA and AA as growth factors *per se*. Their negative relationships with birth dimensions may result from a limited maternal-fetal LCPUFA transfer capacity. Potential effects of 18:1*t* and DGLA on birth dimensions are probably small or non-existing.

Introduction

The essential long-chain polyunsaturated fatty acid (LCPUFA) docosahexaenoic acid (DHA) is an important fetal nutrient, given its active accumulation in the developing brain and retina during the last trimester of gestation [10,121]. Recent research also indicates maternal n-3 LCPUFAs to be important for the programming of fetal growth [74,96]. The role of another LCPUFA, arachidonic acid (AA), in early growth is not clear, since both promoting [110,111] as well as limiting [74,96] potentials have been suggested. Although causality of these associations has not yet been ascertained, it seems important to optimize the neonatal LCPUFA status, since low birth weight is thought to be associated with later occurrence of coronary heart disease and other chronic illnesses [39].

Dietary *trans* fatty acids, mainly formed during industrial hydrogenation of unsaturated edible oils, may interfere with the conversion of the parent essential fatty acids (EFAs) into their LCPUFAs, especially when the parent EFA concentrations are low [22,23]. In this way, *trans* fatty acids may lower the fetal LCPUFA status and, thereby, affect fetal development.

Rump et al. demonstrated that birth weight of term neonates was negatively related to DHA and AA concentrations in umbilical cord plasma phospholipids (PLs), whereas it was positively associated with concentrations of dihomo-γ-linolenic acid (DGLA), the precursor of AA [122]. In contrast, Elias and Innis, observed positive associations between neonatal triacylglycerol AA concentrations and birth weight and birth length, and between cholesteryl ester AA concentrations and birth weight [8]. In addition, no significant associations were found between neonatal plasma *trans* fatty acid concentrations and birth weight and birth length. However, the applicability of this latter study is limited since no corrections were made for relevant covariables. Moreover, Rump et al. restricted their study to birth weight and to cord plasma polyunsaturated fatty acids and did not consider *trans* fatty acids [122]. In addition, they only partially adjusted for potential confounders. Therefore, the present study was conducted to refine and extend these findings with additional birth outcome variables, fatty acid domains and relevant covariables.

Subjects and methods

General design of the study

According to pre-specified inclusion criteria (see below), information with respect to birth outcome variables, fatty acid concentrations and clinical characteristics of eligible infants and their mothers was taken from the database of the Maastricht Essential Fatty Acid Birth cohort (MEFAB database [74]). Associations between selected fatty acid concentrations in PLs of the walls of

umbilical arteries and veins, and of umbilical cord plasma and erythrocytes (independent variables) and birth weight, birth length or head circumference (dependent variables) were studied by unadjusted and multivariable-adjusted linear regression analyses.

Inclusion of participants

The study was executed with data of pregnant women and their newborns who participated in several observational studies, conducted in our institute between 1990 and 1997 in The Netherlands [1,7]. Approval for these studies was obtained from the Medical Ethics Committee of the University Hospital Maastricht and the University of Maastricht, and all participating women gave their written informed consent. Data of infant-mother pairs were included in the present study if the infants were born at term to healthy Caucasian parents after an uncomplicated pregnancy, as detailed before [74]. In addition, PL fatty acid profiles of the four neonatal lipid domains needed to be available. This resulted in 703, 158, 484 and 479 cases available for regression analyses related to cord plasma, erythrocytes, arterial and venous walls, respectively.

Birth dimensions and fatty acid analyses

Birth weight, birth length and head circumference were recorded directly after birth on standardized sheets. Directly after delivery, umbilical cord blood samples were collected, plasma was separated from erythrocytes by centrifugation and after plasma collection the erythrocytes were washed with physiological saline. Furthermore, a piece of umbilical cord was collected and rinsed with saline (NaCl, 0.9 % w/v). Plasma, erythrocytes and umbilical tissue samples were stored under nitrogen at -80 °C until analysis. The fatty acid composition of PLs isolated from plasma, erythrocytes and cord vein and artery walls were determined as described earlier [68,123]. Separation of the various *trans* isomers of octadecenoic acid (C18:1) was incomplete; therefore, they are reported together as 18:1*t*. Fatty acids are expressed as relative contents (% by wt of total amount of identified fatty acids).

Covariables

For reasons explained in the respective references provided below, the following covariables were included in the multivariable-adjusted analyses as potential confounding factors: maternal age [97], height and body mass index (BMI, weight (kg)/height $(m)^2$) at study entry [98], parity [99], smoking and drinking during pregnancy [100], weight increase during pregnancy [101], socio-economic status (income) [102], gestational age [98] and infant sex [103]. Methodological details have been described in detail before [74].

Statistical analyses

Before starting the analyses, outliers (± 4 SD outside the mean) of the dependent variables were removed and the normality of their distributions was checked and confirmed by histograms. Thereafter, associations between various birth outcome measures (the dependent variables; birth weight, birth length and head circumference) and the neonatal fatty acid concentrations of interest, *viz.* DHA, AA, DGLA and 18:1*t*, were analyzed with unadjusted and multivariable-adjusted linear regression analyses. The unadjusted analyses were performed with the same subjects as included in the corresponding multivariable-adjusted analyses. Because of occasionally missing observations, this limited the number of cases for analysis. Therefore, to increase the number of available cases, irrelevant covariables were removed by stepwise backward multivariable-adjusted regression analyses, performed for each fatty acids-birth outcome combination. This procedure has been described in detail before [(74)] and the successive steps were continued until all remaining covariables were either significant or were characterized as confounders. For each particular combination of fatty acids and birth outcome, these various steps were performed with the same dataset. However, since removal of the irrelevant covariables implied less missing values and, consequently, a larger number of cases available for analysis, the ultimate regression analyses were finally repeated with the maximum number of complete cases available for each combination. To check whether the relationships between the dependent and independent variables were comparable for the added cases and the initial study population (a prerequisite for acceptance of this procedure), interaction analyses were performed as detailed before [(74)]. If the added cases were significantly different from the initially included ones, the final model with the larger number of cases could not be accepted. Since these interaction analyses revealed no significant differences between initial and additional cases, all final backward models could be approved.

To determine possible influential cases in the regressions, all data points were checked by calculating their Cook's distance and removed if values were ≥ 1. Such influential data points were not observed, however.

Associations with p-values < 0.010 were considered statistically significant, to correct for multiple testing. A p-value < 0.050 was regarded to indicate a (non-significant) trend. Variables are reported as median (25^{th} - 75^{th} percentile), unless specified otherwise. All statistical analyses were performed using SPSS 11.5 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Table 1 shows the maternal and infant characteristics of the included participants. The relative concentrations (% wt/wt) of DHA, AA, DGLA and 18:1*t* in plasma, erythrocyte and umbilical tissue PLs are given in **table 2**. Outcomes of the regression analyses are shown in **tables 3, 4** and **5.** To reduce the complexity of these tables, only results are shown for those relationships in which significant associations and/or trends were found in either the unadjusted, multivariable-adjusted or final backward analyses. Full results are available on request.

Table 1. Subject characteristics

Maternal characteristics	n	
Age (years)	730	29.0 (26.1 - 31.7)
Height (cm)	698	167 (162 - 170)
BMI at study entry (kg/m^2)	686	22.8 (20.9 - 25.1)
Parity (n) 0 / 1 / ≥ 2	730	551 / 151 / 28
Weight increase during pregnancy (kg)	687	11.8 (9.3 - 14.5)
Socio-economic status (income class)[a]	576	3 (2 - 3)
Smoking during pregnancy (n) no / yes	726	527 / 199
Alcohol during pregnancy (n) no / yes	727	706 / 21
Infant characteristics		
Gestational age (weeks)	730	40.1 (39.3 - 41.1)
Sex (n) male / female	730	392 / 338
Birth weight (g)	728	3323 (437)
Birth length (cm)	629	50.0 (2.2)
Head circumference (cm)	554	34.2 (1.6)

Birth weight, birth length and head circumference were normally distributed and are therefore expressed as mean ± SD. The distributions of the other characteristics were not checked for normality and are, therefore, given as median (25th - 75th percentile).[a] Ranges from minimum (5) to ≥ 2 x modal (1).

Table 2. Relative concentrations (% wt/wt) of fatty acids of interest in neonatal PLs collected in several domains directly after birth

Fatty acids	n	Umbilical plasma	n	Arterial wall	n	Venous wall	n	Erythrocytes
DHA	703	6.07 (5.11 - 7.14)	484	5.11 (4.50 - 5.63)	479	4.89 (4.37 - 5.50)	158	4.65 (4.18 - 5.25)
AA	703	16.99 (15.87 - 18.03)	484	13.68 (12.46 - 15.35)	479	18.19 (17.16 - 19.28)	158	14.23 (13.45 - 14.85)
DGLA	703	5.10 (4.60 - 5.67)	483	1.26 (1.09 - 1.44)	479	1.86 (1.62 - 2.12)	158	2.40 (2.11 - 2.69)
18:1*t*	401	0.11 (0.08 - 0.14)	329	0.14 (0.12 - 0.17)	328	0.10 (0.08 - 0.12)	116	0.10 (0.07 - 0.13)

The relative fatty acid results are expressed as median (25^{th} - 75^{th} percentile). PLs = phospholipids; AA = arachidonic acid; DGLA = dihomo-γ-linolenic acid; DHA = docosahexaenoic acid; 18:1*t* = 18:1*trans* isomers.

Relationship between neonatal 18:1t concentrations and birth dimensions

Unadjusted regression analyses revealed no significant associations between neonatal 18:1*t* concentrations in the four umbilical cord domains and the birth dimensions. However, after full adjustment a significant negative association was observed for the relationship between neonatal 18:1*t* in erythrocyte PLs and birth weight (p = 0.009). The complete model explained 39.2 % of the variability in birth weight (R^2 = .392), 6 % of which was contributed by 18:1*t* (r^2 = .060). In the final backward model the association remained significant and was of the same order of magnitude (p = 0.004, R^2 = .334, r^2 = .058). No other associations or trends were found between 18:1*t* concentrations and birth dimensions.

*Relationship between neonatal DHA concentrations and birth dimensions (**Table 3**)*

After adjustment for all covariables, a significant negative association was found for the relationship between DHA concentrations in umbilical plasma PLs and birth weight. After removal of irrelevant covariables by the stepwise backward procedure, this association remained significant and the model explained 40.8 % of the variability in birth weight, 3.1 % of which was contributed by DHA.
For DHA in umbilical vein wall PLs, unadjusted regression analyses revealed a positive trend with birth length. However, this trend disappeared after full adjustment and after correction for the relevant covariables only.

With respect to DHA in erythrocyte PLs, a negative trend was found in the final backward model for birth weight.

Other associations between birth dimensions and neonatal DHA concentrations were not significant and no additional trends were observed either.

*Relationship between neonatal AA concentrations and birth dimensions (**Table 4**)*

For umbilical plasma PL AA concentrations, a significant negative relationship with birth weight was observed in unadjusted regression analyses. This negative association remained significant after correction for all covariables. The complete model explained 42.4 % of the variability in birth weight and the contribution of AA was 2.4 %. After removal of the irrelevant covariables, the final model explained 40.8 % of the variability in birth weight, and the contribution of AA (1.7 %) remained significant.

Although unadjusted regression analyses did not demonstrate any significant relationship between AA concentrations measured in arterial wall PLs and birth outcome variables, adjustment for covariables revealed significant

Table 3. Unadjusted and multivariable-adjusted (backward) regression analyses of the relationships between birth outcome variables and docosahexaenoic acid concentrations in PLs, collected in different neonatal fatty acid umbilical domains directly after birth

Umbilical cord domain	Birth outcome	Unadjusted (no covariables included)				Multivariable-adjusted model (all covariables included)[a,b]				Final multivariable-adjusted backward model with (only relevant covariables included)[a]						
		n	R^2	B	p	R^2	B	r^2	p	n	R^2	B	r^2	p	95 % CI (B)	
															Low	high
Plasma	BW[c]	291	.001	-8.767	.644	.424	-65.21	.033	*.000*	366	.408	-63.23	.031	*.000*	-92.13	-34.32
Venous wall	BL[d]	189	.028	0.392	.021	.331	0.215	.004	.294	252	.334	0.225	.005	.180	-0.105	0.555
Erythrocytes	BW[e]	90	.001	-15.66	.762	.392	-65.13	.015	.188	110	.334	-93.82	.030	.036	-181.6	-6.071

BW = birth weight; BL = birth length; HC = head circumference; PLs = phospholipids. R^2 = coefficient of determination of total model; r^2 = square of the semi-partial correlation coefficient of fatty acid concerned; B = regression coefficient of fatty acid of interest; p = p-value of fatty acid concerned; CI = confidence interval. [a] The total model p-values of the (final) multivariable-adjusted analyses were all < 0.000, except for Erythrocytes-BL (.011 and .003, for respectively the multivariable and the backward model). [b] Same cases as included in unadjusted model. Relevant covariables in final backward model: [c] maternal height, BMI at study entry, parity, alcohol during pregnancy, weight gain during pregnancy, gestational age and infant sex. [d] maternal height, alcohol during pregnancy, weight gain during pregnancy, gestational age and infant sex. [e] gestational age, body mass index (BMI) at study entry, parity and weight increase during pregnancy. [#] maternal height, BMI at study entry, parity, smoking during pregnancy, alcohol during pregnancy, weight increase during pregnancy, socio-economic status and gestational age. [g] maternal height, alcohol during pregnancy, weight increase during pregnancy, gestational age and infant sex. [h] maternal height, parity, gestational age and infant sex. [i] maternal age, maternal height, BMI at study entry, parity, smoking during pregnancy, weight increase during pregnancy, socio-economic status, gestational age and infant sex. [j] maternal age, maternal height, parity, gestational age and infant sex. [k] maternal height, BMI at study entry, parity, alcohol during pregnancy, weight increase during pregnancy and infant sex. *Italic numbers* refer to a significant relationship ($p < 0.010$), $0.010 \leq p < 0.050$ refer to a non-significant trend.

Table 4. Unadjusted and multivariable-adjusted (backward) regression analyses of the relationships between birth outcome variables and arachidonic acid concentrations in PLs, collected in different neonatal fatty acid umbilical domains directly after birth

Umbilical cord domain	Birth outcome	Unadjusted (no covariables included)				Multivariable-adjusted model (all covariables included)[a,b]				Final multivariable-adjusted backward model with (only relevant covariables included)[a]						
		n	R^2	B	p	R^2	B	r^2	p	n	R^2	B	r^2	p	95 % CI (B)	
															low	High
Plasma	BW[c]	291	.038	-48.02	*.001*	.424	-44.63	.024	*.001*	366	.408	-36.89	.017	*.002*	-59.86	-13.93
Arterial wall	BW[#]	225	.010	-20.91	.127	.440	-61.97	.042	*.000*	225	.433	-65.52	.048	*.000*	-96.09	-34.95
	BL[g]	187	.002	-0.051	.502	.357	-0.295	.036	*.002*	251	.329	-0.232	.023	*.005*	-0.392	-0.072
	HC[h]	166	.016	-0.093	.100	.292	-0.187	.029	.014	226	.246	-0.157	.021	.015	-0.284	-0.030
Venous wall	BW[i]	226	.021	-28.00	.030	.447	-49.81	.040	*.000*	226	.440	-50.54	.041	*.000*	-75.87	-25.20
	HC[j]	168	.024	-0.110	.047	.274	-0.135	.021	.040	228	.244	-0.128	.017	.026	-0.241	-0.015
Erythrocytes	BL[k]	71	.083	-0.485	.015	.374	-0.571	.064	.021	87	.286	-0.436	.043	.035	-0.841	-0.031

For explanations of symbols, see **table 3**.

negative associations between neonatal AA levels and birth weight and birth length. The final multivariable models (with relevant covariables included only) explained 43.3 and 32.9 % of the variability in birth weight and birth length, respectively. The contributions of AA were 4.8 % (for birth weight) and 2.3 % (for birth length). For the association between arterial wall AA concentrations and head circumference, a negative trend was found after full adjustment. The complete model explained 29.2 % of the variability in head circumference with an almost significant contribution of 2.9 % of AA. After adjustment for only the relevant covariables results remained the same.

In unadjusted regression analyses negative trends were observed for the associations between neonatal AA concentrations measured in umbilical vein PLs and birth weight and head circumference. After entering all covariables, these trends became clearly stronger but only for the association with birth weight it became significant. The complete model explained 44.7 % of the variability in birth weight and the contribution of AA was 4.0 %. In the final backward model, results were comparable.

In erythrocyte PLs, only a trend was observed between neonatal AA concentrations and birth length in the unadjusted and multivariable-adjusted models, which remained after the stepwise backward procedure.

No other associations between birth dimensions and neonatal AA concentrations showed trends or were significant.

*Relationship between neonatal DGLA concentrations and birth dimensions (**Table 5**)*

In unadjusted analyses only a positive trend was found for the association between birth weight and DGLA concentrations measured in plasma PLs. After adjustment for all covariables as well as for the relevant covariables only, this trend was lost.

In arterial walls, positive trends were observed for the DGLA associations with birth weight and birth length in the multivariable-adjusted models. Only the trend for birth weight remained after removal of irrelevant covariables by backward regression analysis.

Other associations between birth outcome variables and neonatal DGLA concentrations were not observed.

Table 5. Unadjusted and multivariable-adjusted (backward) regression analyses of the relationships between birth outcome variables and dihomo-γ-linolenic acid concentrations in PLs, collected in different neonatal fatty acid umbilical domains directly after birth

Umbilical cord domain	Birth outcome	Unadjusted (no covariables included)				Multivariable-adjusted model (all covariables included)[a,b]				Final multivariable-adjusted backward model with (only relevant covariables included)[a]						
		n	R^2	B	p	R^2	B	r^2	p	n	R^2	B	r^2	p	95 % CI (B)	
															low	High
Plasma	BW[c]	291	.014	56.10	.047	.424	9.494	.000	.715	366	.408	8.830	.000	.704	-36.77	54.43
Arterial wall	BW[#]	225	.009	151.9	.157	.440	220.9	.012	.036	225	.433	220.6	.012	.036	14.24	426.9
	BL[g]	187	.012	0.874	.135	.357	1.304	.017	.034	251	.329	0.968	.009	.069	-0.076	2.012

For explanations of symbols, see **table 3**.

Discussion

In the present study, we investigated in an infant-mother birth cohort the associations between several birth dimensions and selected fatty acid concentrations in four neonatal domains. These fatty acids are considered to reflect the prenatal exposure of the fetus, since it was observed that fetuses do not possess a different EFA status than infants directly after birth at a comparable gestational age [124]. For DGLA no significant associations were observed. Umbilical plasma PL DHA and AA concentrations appeared negatively related to birth weight, just like AA concentrations in the PLs of arterial and venous umbilical walls. For umbilical erythrocyte PL concentrations of 18:1*t*, the main industrially produced *trans* unsaturated fatty acid present in the diet, also a significant negative association was found with birth weight. Birth length was only significantly associated (in a negative way) with AA concentrations in cord artery wall PLs.

For AA these results are not in line with the general opinion that this LCPUFA stimulates fetal growth. However, this opinion is based on studies in *preterm* infants [110,111]. Studies in *term* babies gave inconsistent results [8,122]. Thus, Elias and Innis observed no significant associations between plasma PL AA concentrations and birth weight and birth length [8]. Interestingly, significant *positive* associations were found between infant plasma cholesteryl ester AA and birth weight and between infant triacylglycerol AA and birth weight and birth length. For the other LCPUFAs no significant results were seen. However, in that study no corrections were made for potential confounders, which could have influenced the study outcomes. Rump and coworkers showed in their study significant negative relations between AA and DHA concentrations in umbilical cord plasma PLs and birth weight [122]. These results are in line with our findings, which could be expected since we used the same database (although extended) as Rump et al. However, we included the fatty acids of interest simultaneously, which enabled an additional correction for their mutual interactions (see below). Furthermore, we included more potential confounders and our analyses were broadened by including fatty acids measured in different umbilical domains.

In the present study, 18:1*t* levels in neonatal PLs (mainly elaidic acid, but including some minor positional isomers also) were negatively associated with most LCPUFA concentrations (data not shown, but available on request). Interestingly, the neonatal *trans* status was hardly associated with birth dimensions, since in our multivariable-adjusted analyses only one significant (negative) association was observed (between 18:1*t* in erythrocyte PLs and birth weight). In the uncorrected study of Elias and Innis, no associations were found between *trans* fatty acids in cord plasma of term infants and birth weight and birth length [8]. On the other hand, Koletzko observed significant negative associations between birth weight and *trans* fatty acid concentrations measured

in various neonatal plasma lipid fractions [22]. However, the latter study was performed in *preterm* infants, which may explain the difference with the results of Elias and Innis. Furthermore, in both studies no corrections were made for any possible confounder. Van Houwelingen and Hornstra observed significant negative correlations between 18:1n-9*t* concentrations in umbilical artery wall PLs and birth weight and head circumference, but this was a relatively small study (n = 37) and correction for potential confounders was incomplete [125]. Also several animal studies have been reported and, overall, no adverse effects of *trans* fatty acids on fetal growth were observed [17,126]. These rather inconsistent results indicate that under the present dietary conditions any potential effect of dietary *trans* unsaturated fatty acids on birth outcome is either small or non-existing.

A strong aspect of our study is that fatty acid concentrations were measured in several umbilical cord domains, reflecting the fetal LCPUFA status over various gestational periods. Thus, cord plasma PLs reflect the LCPUFA availability at the very end of gestation, whereas the vessel wall and erythrocyte PLs, with presumably a lower turnover than plasma PLs [127], represent a longer-term reflection of the fetal LCPUFA status during pregnancy. Furthermore, use of the MEFAB database enabled the selection of a relatively large number of neonatal and maternal covariables. On the other hand, this was an observational study and, therefore, residual confounding can not be excluded.

Relative high drop-out rates in cohort studies due to missing values can bias the results. The high drop-out rate in the present study, especially in the erythrocytes domain, is mainly the consequence of the lower number of fatty acid analyses performed for this domain. However, no major effects of this high drop-out rate on the results were observed for the mean birth dimensions and the median fatty acid values. Even for the maximum drop-out rate the group means of the three birth dimensions differed less than 3 % (ranging from 0.9 to 2.7 %) for the cases who did or did not participate in the regression analyses. It is unlikely that these small birth outcome differences are of clinical relevance. For the fatty acids DGLA, AA, DHA and 18:1*t* these differences were between 2.5 to 4.4 %, averaged over the four fatty acid domains. These differences are relatively small and well within the reproducibility range of our fatty acid analysis (in plasma PL, coefficients of variations were 11.7, 6.1, 9.0 and 29.9 % for DGLA, AA, DHA and 18:1t, respectively).

As mentioned before, we included all four selected fatty acids in the regression models simultaneously, thereby taking into account their mutual metabolic interactions [2,95]. Furthermore, fatty acid contents are reported in relative concentrations and any change in the concentration of one fatty acid will result in a change in the relative concentrations of the other fatty acids included in the analysis. Although correlation coefficients between the selected fatty acids in the several umbilical domains varied between 0.082 and 0.796, the

multicollinearity checks revealed a tolerance value of > 0.1 and a variance inflation factor of < 10, which allowed us to make this decision [114].

Fetuses depend on their mothers to obtain the LCPUFAs needed for optimal development, as is supported by the positive correlations between maternal and fetal LCPUFA concentrations during pregnancy [124,128] and between maternal and neonatal LCPUFA concentrations at birth [6,7,85]. Therefore, maternal plasma fatty acids measured during pregnancy can be taken to reflect the LCPUFA status of the children during the prenatal period also. In two previous studies, associations between maternal fatty acid concentrations, measured during pregnancy, and birth weight, birth length or head circumference have been investigated [74,96]. In both studies significant negative associations were observed between maternal AA concentrations and some birth outcomes, which is in line with results of the present study. For DHA concentrations, however, contrasting study results were found when birth dimensions were related to maternal (positive associations) or to neonatal fatty acid levels (negative association). The negative association in the present neonatal study might be explained by the assumption that the placental LCPUFA transfer is limited. As a result, a larger fetus might have less LCPUFAs available per mass unit than a smaller fetus. Further studies are needed to test this hypothesis.

In the present study the unadjusted and multivariable-adjusted analyses were performed with the same number of complete cases (all (co)variables available). In general, stronger associations were observed when unadjusted regression analyses were executed with the maximum number of cases available, thereby increasing the power of the analyses. For DGLA in cord plasma PLs this resulted in a significant positive association ($p = 0.001$) with birth weight, as observed before by Rump and coworkers [122]. In addition, birth length appeared negatively associated with AA in cord plasma PLs ($p < 0.000$). No significant associations were observed in the unadjusted analyses of the relationships between neonatal DHA concentrations and birth weight, but significant positive relationships were found with birth length ($p < 0.005$ for 3 lipid domains). Although this is in line with the positive trend we observed in the initial unadjusted regression analysis for vein wall DHA (see **table 3)**, this trend disappeared after adjustment for relevant covariables. Therefore, it seems rather unlikely that DHA has a promoting effect on birth length. Increasing the power of the unadjusted regression analyses by including all available cases did not result in other major differences (results not shown).

In conclusion, DHA and AA concentrations measured in various umbilical domains and considered to reflect fetal LCPUFA availability during late gestation are mainly negatively related to birth weight and birth length. Although these fatty acids, as essential membrane constituents, are required to allow fetal growth to take place, our results seems to preclude their role as growth factors *per se*. The negative relationships observed may result from a limited

maternal-fetal LCPUFA transfer capacity. Associations with birth dimensions are weak or non-existing for 18:1*t* and DGLA.

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Chapter 6

Dietary arachidonic acid dose-dependently increases the arachidonic acid concentration in human milk

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Abstract

Lactation hampers normalization of the maternal arachidonic acid (AA) status, which is reduced after pregnancy and can further decline by the presently recommended increased consumption of n-3 long-chain polyunsaturated fatty acids (n-3 LCPUFAs). This may be unfavorable for breast-fed infants, because they also require an optimum supply of n-6 LCPUFAs. We therefore investigated the LCPUFA responses in nursing mothers upon increased consumption of AA and n-3 LCPUFAs. In a parallel, double-blind, controlled trial, lactating women received for 8 weeks (wks) no extra LCPUFAs (Control group, n = 8), 200 (low AA group, n = 9) or 400 (high AA group, n = 8) mg/d AA in combination with n-3 LCPUFAs (320 mg/d docosahexaenoic acid (DHA), 80 mg/d eicosapentaenoic acid (EPA) and 80 mg/d other n-3 fatty acids), or this dose of n-3 LCPUFAs alone (DHA + EPA group, n = 8). Relative concentrations of AA, DHA and sums of n-6 and n-3 LCPUFAs were measured in milk total lipids (TLs) and erythrocyte phospholipids (PLs) after 2 and 8 wks and changes were compared by ANCOVA. The combined consumption of AA and n-3 LCPUFAs caused dose-dependent elevations of AA and total n-6 LCPUFA concentrations in milk TLs and did not significantly affect the DHA and total n-3 LCPUFA increases caused by n-3 LCPUFA supplementation only. This latter treatment did not significantly affect breast milk AA and total n-6 LCPUFA concentrations. AA and DHA concentrations in milk TLs and their changes were strongly and positively correlated with their corresponding values in erythrocyte PLs (r^2 = 0.27 - 0.50; $p \leq 0.002$). We thus concluded that the consumption by lactating women of AA in addition to extra n-3 LCPUFAs dose-dependently increased the AA concentration of their milk TLs.

Introduction

Arachidonic acid (AA, 20:4n-6) and docosahexaenoic acid (DHA, 22:6n-3) are the most abundant long-chain polyunsaturated fatty acids (LCPUFAs) in the membrane phospholipids (PLs) of neural tissues, including brain [129-131]. Especially during rapid neurodevelopment of the fetus in the last trimester of pregnancy and in the early postnatal period, AA and DHA accumulate in large amounts in neural tissues [66], where they serve as important structural and functional components in the development of neural and synaptic networks [132].

Although newborn infants are capable of synthesizing AA and DHA from precursor fatty acids, this capacity seems insufficient to meet the high demands of the developing tissues [29,30]. Consequently, for these LCPUFAs, infants largely depend on an adequate dietary supply, preferably from breast milk.

Pregnancy is associated with a reduction in the relative concentrations of AA and DHA (after an initial increase) in maternal plasma PLs, because the maternal-fetal LCPUFA transfer is insufficiently compensated for by increased maternal LCPUFA consumption [133,134]. During lactation, women continue the transfer of their own LCPUFAs to their infants, but this does not compromise the restoration of their relative plasma PL AA concentrations [5]. The relative DHA concentrations in plasma PLs of lactating mothers, however, become lower than those of non-lactating mothers and also lower than those before conception [27]. To support maternal DHA concentrations and to stimulate the n-3 LCPUFA intake of their infants, an international working party recently recommended increased maternal consumption of n-3 LCPUFAs during pregnancy and lactation of ~200 mg/d [135]. Because recommendations for the general population are noticeably higher [136,137], the n-3 LCPUFA consumption of pregnant and lactating women can be expected to increase considerably in the near future. However, an increase in the consumption of n-3 LCPUFAs often causes a concomitant decrease of circulating n-6 LCPUFA concentrations and of AA in particular [9,138], although this is not a consistent finding [139]. AA is considered important for fetal and infant growth and development [31,32], possibly because of its multiple physiological function such as involvement in eicosanoid synthesis [140], diacylglycerol cell-signaling pathways [141] and transcription regulation of fat metabolizing enzymes [142].

Observational studies indicate that AA concentrations in human milk are less variable than DHA levels [143], which may be due to its putative resistance to changes in the consumption of AA and its precursors [144-146]. However, data from supplementation studies are limited. We thus decided to further investigate the responsiveness of human milk to the additional supply of n-3 and n-6 LCPUFAs. For this purpose, we first investigated the impact of the added consumption of 400 mg/d DHA + eicosapentaenoic acid (EPA) on the concentrations of AA and other LCPUFAs in breast milk total lipids (TLs) of lactating mothers. Subsequently, we studied the influence of co-consumption of

200 or 400 mg/d AA. To check the effects of these supplemental LCPUFAs on the general LCPUFA status of the women, we also measured LCPUFA concentrations in their erythrocyte PLs.

Subjects and Methods

Subjects

A total of 52 healthy women in week (wk) 34 or 35 of pregnancy and intending to breast-feed their babies for at least 3 months were recruited at the University Hospital Maastricht and the Atrium Hospital Heerlen (both The Netherlands) through their obstetricians or midwives during their regular checks and by public relation activities. Further inclusion criteria for study entry were: pregnant for the first, 2nd or 3rd time; apparently healthy; pre-pregnancy BMI between 18 and 27 kg/m^2; no vegetarian lifestyle, fish consumption < 2 times/wk, no use of supplements or products rich in LCPUFAs and an alcohol consumption of ≤ 63 g/wk; no use of medication, drugs or supplements (iron and folic acid allowed); smoking ≤ 5 cigarettes/d; no participation in another study < 2 months ago. Reasons for exclusion after enrolment were delivery before wk 37 or after wk 43 of gestation, failure to comply with the demands of the study and suffering from an adverse event that might impair the reliability of the results. Subjects donated a blood sample in wk 36 of pregnancy to check their essential fatty acid status and were randomly assigned to 1 of the 4 test groups at wk 3 after delivery. The study was approved by the Medical Ethics Committee of the University Hospital Maastricht and conducted in accordance with the Helsinki Declaration. A written informed consent was obtained from each subject before enrolment.

Dietary supplementation

The subjects were asked to consume twice per day 200 mL of 1 of the 4 milk powder-based test drinks (in the morning and in the evening). For a 200-mL test product, a portion of 38 g powder was dissolved in a glass (~180 mL) of tepid water (**Table 1**). Per 38 g, all powders (Friesland Coberco Dairy Foods) contained ~5.7 g protein, 21.5 g carbohydrate, 5.5 g fat and vitamins and minerals. The control group received a control product without added LCPUFAs. Subjects allocated to the 3 treatment groups (DHA + EPA, low AA and high AA groups) received the control product enriched with n-3 LCPUFAs from Dry n-3 (BASF Health and Nutrition). The test products of the low and high AA groups contained also AA from Optimar single-cell oil (DSM Food Specialties). The consumption of 2 portions of the test drinks per day resulted in an additional daily intake of 320 mg DHA, 80 mg EPA and 80 mg of other n-3

fatty acids (all 3 treatment groups), and 200 mg/d AA (low AA group) or 400 mg/d AA (high AA group). Treatment compliance was assessed on the difference between distributed full and returned (partly) empty cans and expressed in percentage.

Table 1. Composition of the 4 test products per portion[1]

	Control group[2]	DHA + EPA group[3]	Low AA group[4]	High AA group[5]
	Unit/200 mL			
Milk protein (g)	5.5	5.9	5.7	5.6
Carbohydrate[6] (g)	22.4	20.7	21.2	21.7
Total fat[7] (g)	5.6	5.5	5.4	5.4
Saturated fat (g)	2.6	2.5	2.5	2.4
Unsaturated fat (g)	2.9	2.9	2.9	3.0
18:2n-6 (LA) (mg)	622	633	587	543
20:4n-6 (AA) (mg)	-	-	100	200
18:3n-3 (ALA) (mg)	89	93	93	93
20:5n-3 (EPA) (mg)	-	40	40	40
22:6n-3 (DHA) (mg)	-	161	161	161

[1] One portion = 38 g powder dissolved in ~180 mL water yielding ~200 mL test product; 2 portions were consumed each day. [2] Intake per day: no LCPUFA. [3] Intake per day: 320 mg DHA + 80 mg EPA + 80 mg other n-3 fatty acids. [4] Intake per day: as DHA + EPA group + 200 mg AA. [5] Intake per day: as DHA + EPA group + 400 mg AA. [6] 33 % Lactose, 50 % sucrose syrup and 17 % sucrose.
[7] 87 % Fat blend FFOO (Friesland Coberco Dairy Foods), 12 % medium-chain triglyceride oil and 1 % milk fat.

Study visits and sample collections

At wk 3 post-delivery (baseline) and 2 and 8 wks later (i.e. at wk 5 and 11 post-delivery), the women were visited at their homes to assess their well-being and that of their children, to check the potential occurrence of adverse events and to determine their treatment compliance. During these visits, blood from a forearm vein was sampled into EDTA-containing tubes. Moreover, the mothers had been asked beforehand to collect, midway during the morning feeding, ~10 mL milk from each breast by manual expression into 2 collection tubes and store

these in a refrigerator. The blood and breast milk samples were kept in a cool-box during transportation to the laboratory.

Sample processing and fatty acid analysis

Blood was processed and erythrocytes and plasma were stored until analysis as detailed before [147]. The breast milk samples from both tubes were pooled and redistributed over 2 new tubes, which were tightly closed under nitrogen and stored at -80 °C until analysis.

All erythrocyte samples of a given subject were analyzed within the same analysis to ensure uniformity of the analytical conditions. The same strategy was applied to the analysis of the breast milk samples.

Lipids were extracted, PLs isolated and fatty acid methyl esters prepared as described earlier [147]. Fatty acid compositions were analyzed by capillary gas chromatography with flame-ionization detection using a polar and a non-polar column (50-m BPX70 polar column, 0.22 x 0.25 µm and 50-m BP1 non-polar column, 0.22 x 0.10 µm, SGE, Bester BV) and optimized injection, oven, and detection temperatures [96]. Helium was used as carrier gas.

Fatty acids were quantified by the amount of internal standard recovered and expressed in absolute concentrations (g/L erythrocyte suspension or breast milk) and relative concentrations as g/100 g of total identified fatty acids in erythrocyte PLs and milk TLs.

In total, 45 and 42 different fatty acids were identified and quantified in milk TLs and erythrocyte PLs, respectively. For this study, we concentrated on the following fatty acids and fatty acid combinations: AA (20:4n-6), DHA (22:6n-3), sum of n-6 LCPUFAs (∑n-6 LCPUFAs: calculated as the sum of 20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6) and sum of n-3 LCPUFAs (∑n-3 LCPUFAs: calculated as the sum of 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3). Based on 8 samples of a single milk pool that were analyzed together with the study samples, we calculated coefficients of variation (CV) values of 1.16 % for AA and 0.66 % for DHA. By means of 6 erythrocyte pool samples, we determined CV values of 0.78 % for AA and 1.07 % for DHA, respectively.

Statistics

We checked fatty acid data for the presence of outliers by using the Studentized deleted residual test [148]. Subsequently, variables not following normal distribution were log-transformed.

Differences between the 4 groups, with respect to the clinical variables and baseline fatty acid concentrations, were tested for statistical significance by ANOVA with Tukey's continuation to locate differences, if any. For variables not normally distributed, the Kruskal-Wallis test was applied and continued by a Bonferroni-corrected Mann-Whitney test. ANCOVA was used to compare the

differences between groups regarding selected fatty acid concentrations after 2 and 8 wks of intervention. Baseline values of the respective variables were used as covariates in these ANCOVAs. Because the linoleic acid (LA) status is known to affect uptake and/or incorporation of n-3 LCPUFAs upon supplementation [149,150], LA concentrations of erythrocyte PLs measured at baseline (ranging between 8.47 and 11.08 g/100 g) were applied as covariates for these ANCOVAs as well. Significant differences between the groups were located by Tukey's post hoc testing. Within each group, changes in fatty acid concentrations between baseline and after 2 and 8 wks of supplementation were tested for significance by Student's paired samples t-test (normal distribution) or Wilcoxon's signed-rank test (distribution not normal). Differences were considered significant at $p < 0.050$, unless indicated otherwise. These statistics were performed with and without outliers.

Relationships between AA and DHA concentrations in breast milk TLs and erythrocyte PLs at baseline and their changes after 2 and 8 wks of intervention were studied in the combined data of all 4 test groups by linear regression analyses. Normal distributions of the residuals were required for acceptance of the regression outcomes.

All statistical analyses were performed using SPSS 12.0.1 for Windows (SPSS Inc., Chicago, USA). Results are expressed as group means ± SD, unless otherwise specified.

Results

Characteristics of the study population at baseline

Fifty-two subjects were randomly allocated to the 4 test groups. Thirty-four subjects completed the study [n = 9 (control and low AA groups) or 8 (DHA + EPA and high AA groups)], whereas 18 withdrew. Reasons for withdrawal were mainly related to problems with breast-feeding or illness of the child. In general, compliance was adequate (88-98 %) and did not differ between the groups. However, 1 subject from the control group was excluded from all data analyses because of an irregular intake of the supplement.

Baseline characteristics did not differ among the groups with the exception of age at delivery, which was significantly lower in the DHA + EPA group than in other groups (**Table 2**).

Table 2. Baseline characteristics of the 4 test groups[1]

Characteristic	Control group	DHA + EPA group	Low AA group	High AA group	p
n	*8*	*8*	*9*	*8*	
Age at delivery (years)	33.9 ± 1.1	28.8* ± 3.2	32.3 ± 3.8	31.9 ± 2.6	0.008
Pre-pregnancy weight (kg)	65.5 ± 7.4	70.2 ± 7.2	63.3 ± 6.4	66.6 ± 6.9	0.251
Height (m)	1.70 ± 0.07	1.71 ± 0.06	1.69 ± 0.05	1.71 ± 0.07	0.829
Pre-pregnancy BMI (kg/m^2)	22.6 ± 2.5	23.9 ± 1.8	22.1 ± 1.7	22.9 ± 3.0	0.492
Systolic blood pressure (mm Hg)	114.3 ± 7.4	120.4 ± 10.5	109.4 ± 8.3	113.6 ± 5.9	0.077
Diastolic blood pressure (mm Hg)	68.2 ± 6.6	69.3 ± 8.1	64.5 ± 5.7	70.0 ± 3.4	0.280
Pregnancies (n)	1.8 ± 0.9	1.9 ± 0.6	1.9 ± 0.6	1.6 ± 0.5	0.842
Total fatty acids in milk TLs[2] (g/L)	33.7 ± 16.7	38.2 ± 8.6	23.2 ± 7.3	36.5 ± 15.2	0.085
Total fatty aids in erythrocyte PLs[2] (g/L)	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.2	0.625

[1] values are means ± SD. * Different from the other groups (ANOVA followed by Tukey's posthoc test, $p < 0.050$).[2] Week 3 after parturition.

Fatty acid composition of breast milk TLs

At baseline (wk 3 post-delivery), breast milk total fatty acid concentrations did not differ among the groups ($p = 0.085$; **Table 2**). AA, Σn-6 LCPUFA, DHA and Σn-3 LCPUFA concentrations also did not differ significantly among the 4 groups ($p > 0.050$; **Table 3**).

Effect of DHA + EPA consumption on AA and Σn-6 LCPUFA concentrations. As demonstrated by the control group, lactation was associated with significant reductions of the AA ($p = 0.011$ after 2 wks, $p = 0.0004$ after 8 wks) and ∑n-6 LCPUFA ($p = 0.028$ after 8 wks) concentrations (**Figures 1A**, **B**). Comparison between the control and the DHA + EPA groups revealed that the extra consumption of 400 mg/d DHA + EPA did not affect breast milk AA or ∑n-6 LCPUFA concentrations after 2 or 8 wks of lactation ($p > 0.050$; **Table 3**).

Table 3. Selected fatty acid concentrations in milk TLs of lactating women at baseline and after 2 and 8 wks of dietary intervention with different doses of AA and n-3 LCPUFAs[1]

Variable	Time	Control group	DHA + EPA group	Low AA group	High AA group
n		*8*	*8*	*9*	*8*
		g/100 g total fatty acids			
AA	B	0.52 ± 0.03	0.45 ± 0.08	0.50 ± 0.11	0.47 ± 0.08
	2	0.45 ± 0.06^{a}	0.43 ± 0.09^{a,x}	0.53 ± 0.08^{y}	0.55 ± 0.09^{y}
	8	0.41 ± 0.06^{a}	0.40 ± 0.04^{a,x}	0.49 ± 0.10^{y}	0.56 ± 0.07^{z}
∑n-6 LCPUFAs	B	1.12 ± 0.05	1.08 ± 0.08	1.04 ± 0.18	1.01 ± 0.17
	2	1.06 ± 0.10^{a}	1.05 ± 0.12^{a,x}	1.06 ± 0.19^{x}	1.08 ± 0.19^{x}
	8	0.89 ± 0.10^{a}	0.90 ± 0.11^{a,x}	0.99 ± 0.19^{x,y}	1.04 ± 0.14^{y}
DHA	B	0.30 ± 0.06	0.34 ± 0.10	0.30 ± 0.11	0.34* ± 0.19
	2	0.25 ± 0.08^{x}	0.55 ± 0.23^{y}	0.60$^{\#}$ ± 0.26^{y}	0.46 ± 0.13^{y}
	8	0.24 ± 0.08^{x}	0.53 ± 0.13^{y}	0.54 ± 0.17^{y}	0.50 ± 0.14^{y}
∑n-3 LCPUFAs	B	0.64 ± 0.08	0.74 ± 0.15	0.62 ± 0.16	0.68** ± 0.29
	2	0.65 ± 0.20^{x}	0.99 ± 0.35^{x,z}	1.02$^{\#\#}$ ± 0.42^{y,z}	0.80 ± 0.19^{x,z}
	8	0.57 ± 0.17^{x}	0.88 ± 0.18^{y}	0.92 ± 0.26^{y}	0.84 ± 0.22^{y}

[1] Values are means ± SD. Means in a row with superscripts without a common letter a, b or c (ANCOVA, control and DHA + EPA groups) or x, y or z (ANCOVA, DHA + EPA, low AA and high AA groups or all 4 groups) differ, $p < 0.050$. * Includes 1 outlier; results after removal: 0.28 ± 0.10 (n = 7). # Includes 1 outlier; results after removal: 0.52 ± 0.11 (n = 8). ** Includes 1 outlier; results after removal: 0.59 ± 0.10 (n = 7). ## Includes 1 outlier; results after removal: 0.89 ± 0.21 (n = 8). B, baseline.

Effect of low AA and high AA consumption on AA and Σn-6 LCPUFA concentrations. Compared with the consumption of the extra n-3 LCPUFAs only (DHA + EPA group), the additional AA intake increased the AA concentration in breast milk TLs (low AA and high AA groups; $p < 0.050$; **Table 3**). For both AA doses, the effect was already significant after 2 wks and persisted during the entire study period of 8 wks. The effect of the high AA dose was greater than that of the low AA dose at 8 wks ($p < 0.050$).

Although the differences in ∑n-6 LCPUFA concentrations between the 3 experimental groups followed a pattern comparable to the differences in AA, most were not significant ($p > 0.050$). One exception was the concentration after

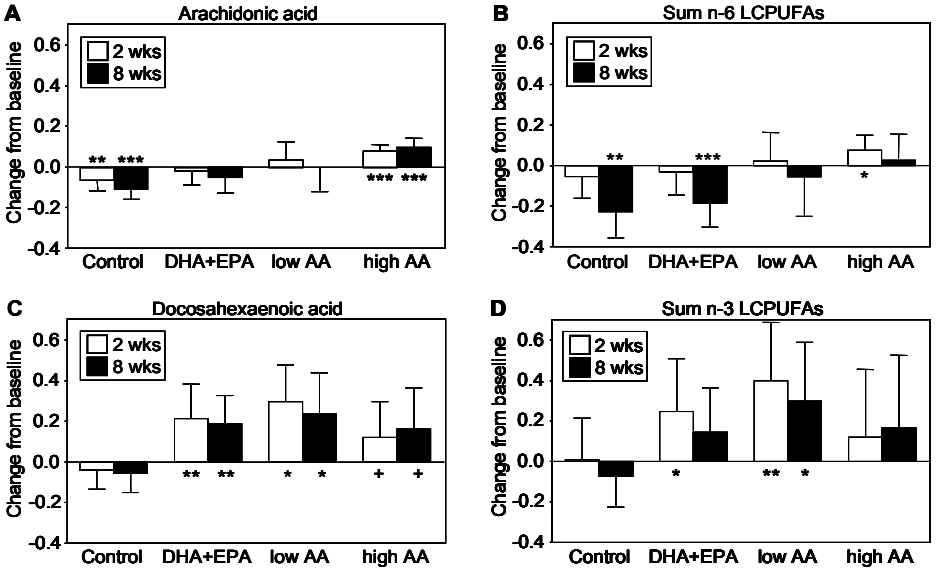

Figure 1. Changes in concentrations (g/100 g fatty acids) of AA (**A**), total n-6 LCPUFAs (**B**), DHA (**C**) and total n-3 LCPUFAs (**D**) in breast milk TLs of lactating women receiving no additional LCPUFAs (control group, n = 8), 320 mg DHA + 80 mg EPA + 80 mg other n-3 fatty acids per day (DHA + EPA group, n = 8), 480 mg n-3 fatty acids + 200 mg/d AA (low AA group, n = 9) or 480 mg n-3 fatty acids + 400 mg/d AA (high AA group, n = 8). Values are means ± SD after 2 and 8 wks of supplementation. Change from baseline (paired samples t-test): + 0.050 ≤ p < 0.100; *0.010 < p ≤ 0.050; **0.001 < p ≤ 0.010; ***p ≤ 0.001.

8 wks in the group receiving the high AA dose, which was higher than that in the women receiving additional n-3 LCPUFAs only (p < 0.050).

Effect of low AA and high AA consumption on DHA and Σn-3 LCPUFA concentrations. In contrast to the n-6 LCPUFA concentrations, the DHA and ∑n-3 LCPUFA concentrations did not differ during the course of lactation (**Figures 1C**, **D**; control group: p = 0.254 and 0.930 (at wk 2), p = 0.148 and 0.224 (at wk 8) for DHA and ∑n-3 LCPUFAs, respectively). Compared with this group, the additional consumption of 400 mg/d DHA + EPA significantly increased the DHA concentration (**Table 3**). This increase was already maximal after 2 wks of intervention and co-administration of 200 or 400 mg/d AA did not significantly affect it after either 2 or 8 wks. This outcome was completely independent of 2 DHA values identified as outliers.

After 8 wks of intervention, results for ∑n-3 LCPUFAs (which also included 2 outliers due to the outlying DHA concentrations) were virtually identical to the DHA outcomes, showing a significant increase after the consumption of additional n-3 LCPUFAs, which was not significantly altered by AA co-

administration. Again, this outcome was independent of inclusion or exclusion of both outliers.

Also after 2 wks, the results for the ∑n-3 LCPUFAs were qualitatively comparable to the findings for DHA. The only exception was the significant increase in ∑n-3 LCPUFA concentration in the low AA group compared with the control group. However, this significant increase could be attributed to an outlier, because after its removal significance was lost.

Fatty acid composition of erythrocyte PLs

At baseline (wk 3 post-delivery), total amounts of fatty acids associated with maternal erythrocyte PLs did not differ among the groups ($p > 0.050$; **Table 2**). The same was true for the relative amounts of selected LCPUFAs and their sums (**Table 4**).

Table 4. Selected fatty acid concentrations in erythrocyte PLs of lactating women at baseline and after 2 and 8 wks of dietary intervention with different doses of AA and n-3 LCPUFAs[1]

Variable	Time	Control group	DHA + EPA group	Low AA group	High AA group
n		*8*	*8*	*9*	*8*
		g/100 g total fatty acids			
AA	B	10.19 ± 0.75	10.00 ± 0.76	9.71 ± 1.10	9.60 ± 0.66
	2	10.20 ± 0.85^{a}	9.92 ± 0.64^{a,x}	10.05 ± 1.15^{y}	10.15 ± 0.66^{y}
	8	10.47 ± 0.93^{a}	9.98 ± 0.55^{a,x}	10.51 ± 0.96^{y}	10.65 ± 0.95^{y}
∑n-6 LCPUFAs	B	14.84 ± 0.96	14.68 ± 1.30	13.93 ± 1.56	14.22 ± 0.99
	2	14.87 ± 0.98^{a}	14.43 ± 1.09^{b,x}	14.14 ± 1.70^{y}	14.63 ± 0.99^{y}
	8	15.00 ± 1.10^{a}	14.13 ± 0.98^{b,x}	14.42 ± 1.27^{y}	14.89 ± 1.46^{y}
DHA	B	4.36 ± 0.43	4.08 ± 0.87	4.11 ± 0.62	4.16 ± 0.73
	2	3.96 ± 0.37^{x}	4.04 ± 0.77^{y,z}	4.15 ± 0.59^{y}	4.02 ± 0.68^{z}
	8	3.41 ± 0.36^{x}	4.35 ± 0.60^{y}	4.26 ± 0.60^{y,z}	3.98 ± 0.47^{z}
∑n-3 LCPUFAs	B	6.74 ± 0.53	6.49 ± 1.04	6.31 ± 0.88	6.34 ± 0.84
	2	6.42 ± 0.44^{x}	6.53 ± 0.98^{y,z}	6.50 ± 0.86^{y}	6.24 ± 0.74^{x,z}
	8	6.17 ± 0.61^{x}	7.00 ± 0.83^{y}	6.70 ± 0.91^{y,z}	6.27 ± 0.58^{x,z}

[1] Values are means ± SD. Means in a row with superscripts without a common letter a, b or c (ANCOVA, control and DHA + EPA groups) or x, y or z (ANCOVA, DHA + EPA, low AA and high AA groups or all 4 groups) differ, $p < 0.050$. B, baseline.

Effect of DHA + EPA consumption on AA and Σn-6 LCPUFA concentrations. As demonstrated by the control group, lactation did not significantly reduce the concentrations of AA and ∑n-6 LCPUFAs compared with baseline values (AA: p = 0.829 after 2 wks and 0.064 after 8 wks; ∑n-6 LCPUFAs: p = 0.782 and 0.315 after 2 and 8 wks, respectively; **Figures 2A**, **B**). The consumption of 400 mg/d DHA + EPA did not significantly affect the AA concentrations after either 2 or 8 wks (comparison between control and DHA + EPA groups; **Table 4**; p > 0.050). However, consumption of the extra n-3 LCPUFAs significantly reduced the ∑n-6 LCPUFA concentrations, both after 2 wks as well as after 8 wks.

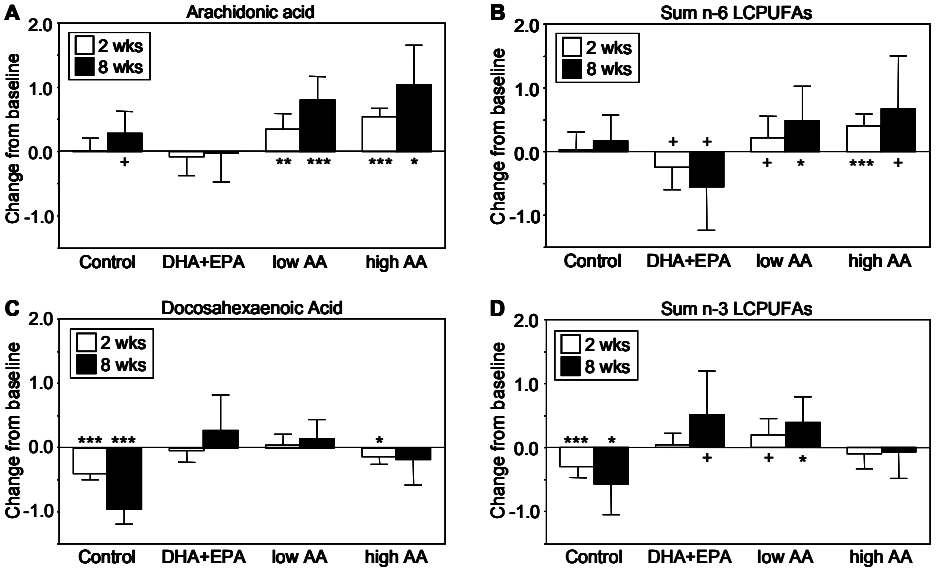

Figure 2. Changes in concentrations (g/100 g fatty acids) of AA (**A**), total n-6 LCPUFAs (**B**), DHA (**C**) and total n-3 LCPUFAs (**D**) in erythrocyte PLs of lactating women receiving no additional LCPUFAs (control group, n = 8), 320 mg DHA + 80 mg EPA + 80 mg other n-3 fatty acids per day (DHA + EPA group, n = 8), 480 mg n-3 fatty acids + 200 mg/d AA (low AA group, n = 9) or 480 mg n-3 fatty acids + 400 mg/d AA (high AA group, n = 8). Values are means ± SD after 2 and 8 wks of supplementation. Change from baseline (paired samples t-test): + 0.050 ≤ p < 0.100; *0.010 < p ≤ 0.050; **0.001< p ≤ 0.010; ***p ≤ 0.001.

Effect of low AA and high AA consumption on AA and Σn-6 LCPUFA concentrations. Compared with the consumption of the extra n-3 LCPUFAs only (DHA + EPA group), co-administration of AA increased the AA concentration (p < 0.050; **Table 4**). For both AA doses, the effect was already significant after 2 wks and persisted during the entire study period of 8 wks. At both points in time, the effect of the high AA dose did not differ from that of the low AA dose (p > 0.050; **Table 4**).

Results for ∑n-6 LCPUFA concentrations were identical to those observed for AA, showing significant increases after 2 as well as 8 wks of intervention that were not significantly different for both AA doses (**Table 4**).

Effect of low AA and high AA consumption on DHA and Σn-3 LCPUFA concentrations. In the control group, receiving no additional LCPUFAs, the DHA concentration strongly decreased during lactation ($p < 0.001$ after 2 wks, $p < 0.001$ after 8 wks; **Figure 2C**). Comparable changes were observed for total n-3 LCPUFAs, although less pronounced ($p < 0.001$ and 0.0126 after 2 and 8 wks, respectively; **Figure 2D**).

Compared with the control group, the additional consumption of 400 mg/d DHA + EPA significantly increased the DHA concentration after 2 and 8 wks of intervention (**Table 4**). This effect was not significantly altered by co-administration of 200 mg/d AA. Co-consumption of 400 mg/d AA, however, attenuated this DHA increase, although the difference with the control group was still significant. Co-consumption of the high AA dose resulted in a significant difference compared with the low AA dose after 2 wks and compared with the DHA + EPA group after 8 wks.

Results for the Σn-3 LCPUFAs were largely comparable (**Table 4**). After 2 and 8 wks, the daily administration of 400 mg DHA + EPA significantly increased the ∑n-3 LCPUFA concentration compared with the control group. Co-administration of 200 mg/d AA did not significantly influence this effect. In contrast, the daily co-administration of 400 mg AA considerably reduced the ∑n-3 LCPUFA increase, as demonstrated by the absence of a significant difference with the control group but significant differences with the DHA + EPA group (at 8 wks only) and the low AA group (at 2 wks).

Relationships between breast milk TLs and erythrocyte PLs with respect to AA and DHA concentrations and their changes.

When data of all 4 test groups were combined, linear regression analyses revealed significant relationships between erythrocyte PLs and breast milk TLs for AA and DHA concentrations at baseline ($p < 0.001$; **Figures 3A**, **B**) as well as after 2 and 8 wks of LCPUFA consumption (data not shown). Significant relationships between both domains were also observed for changes in AA and DHA concentrations both over the first 2 wks of intervention ($p < 0.001$; **Figures 3C**, **D**) as well as over the entire intervention period of 8 wks [$p = 0.002$ (AA change), $p = 0.001$ (DHA change); **Figures 3E**, **F**].

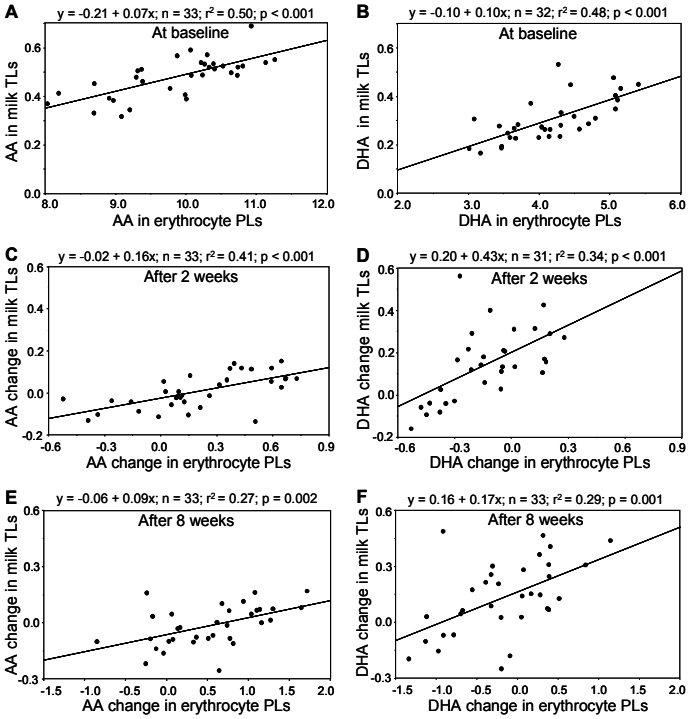

Figure 3. Correlations between fatty acid concentrations in breast milk TLs and erythrocyte PLs of lactating women. Results are presented for AA (**A**) and DHA (**B**) at baseline and for changes in AA and DHA concentrations after 2 (**C**, **D**) and 8 (**E**, **F**) wks of supplementation with preparations containing either or not (control group) additional LCPUFAs. Fatty acid concentrations and concentration changes are given in g/100 g fatty acids.

Discussion

Because of their multiple health benefits, it can be expected that the intake of n-3 LCPUFAs will increase in the general population and in pregnant and lactating women, in particular. Elevated n-3 LCPUFA consumption often coincides with a decreased AA status of an individual. In pregnant and lactating women, however, this may not be desirable, because AA is considered essential for fetal and neonatal development [31,32]. Hence, in a group of lactating women we investigated the effects of the extra consumption of 320 mg/d DHA and 80 mg/d EPA on the n-6 and n-3 LCPUFA concentrations in their milk over a period of 8 wks. In addition, we studied the effects of 200 and 400 mg/d AA added to these n-3 LCPUFAs. To monitor changes in the general LCPUFA status of the participating women, the response of the LCPUFA concentrations were measured in erythrocyte PLs.

Compared with the control product without added LCPUFAs, the daily consumption of 400 mg extra n-3 LCPUFAs did not significantly alter the AA and n-6 LCPUFA concentrations in breast milk TLs (**Table 3**). Consistent with earlier reports [151-153], we observed that the milk DHA concentrations significantly increased within 2 wks upon the intake of 320 mg/d DHA and 80 mg/d EPA. The extra consumption of 200 or 400 mg/d AA significantly increased the AA concentration in breast milk TLs both after 2 and 8 wks of intervention in a dose-dependent manner. However, it required > 2 wks before the dose effect became significant, which might have been due to the rather small sizes of our test groups. The Σn-6 LCPUFA concentrations of breast milk TLs also increased upon AA consumption. This effect was only significant for the high AA dose and an intake period of > 2 wks (**Table 3**).

Based on indirect evidence, it has been suggested that maternal AA intake influences the AA content of breast milk only to a minor extent [144,146]. However, the effect of AA consumption on breast milk AA concentration has hardly been investigated. The only study we are aware of was performed by Smit et al. [145], who supplied lactating women with 300 mg AA either alone or in combination with 400 mg DHA and 110 mg EPA per day for 1 week . No significant effects were found on the AA concentration of breast milk TLs, which was recently attributed to the short study period [154].

In general, the LCPUFA responses in breast milk TLs were in good agreement with those observed in erythrocyte PLs (**Table 4**), but not in all aspects. Although consumption of extra n-3 LCPUFAs did not significantly affect AA concentrations in erythrocyte PLs, the Σn-6 LCPUFA concentrations progressively decreased. This decline appeared mainly attributed to the significantly decreasing concentrations of adrenic acid (AdrA, 22:4n-6; results not shown, but available on request), the elongation product of AA.

As expected, the DHA and Σn-3 LCPUFA concentrations increased significantly in the red blood cell PLs upon the consumption of the extra n-3 LCPUFAs (**Table 4**). Interestingly, the concomitant intake of AA attenuated this effect considerably, which indicates that AA may compete with DHA for incorporation into erythrocyte membrane PLs. Such a competition likely explains results, reported by Nelson et al. [155], that the daily consumption of 1.5 g AA for 50 d significantly lowers the DHA content of erythrocyte PLs. In our study, the extra AA also raised the AA status of the women, as demonstrated by the AA increase in their erythrocyte PLs.

During the entire study period, breast milk TL concentrations of AA and DHA as well as their diet-induced changes were positively and significantly correlated with AA and DHA concentrations and changes in erythrocyte PLs (see **figure 3**). Dietary AA and DHA are generally acknowledged to directly affect the AA and DHA concentrations in erythrocyte PLs and the correlation with concentrations and changes in milk TLs could suggest that they may also directly influence the breast milk concentrations of these LCPUFAs.

Remarkably, the regression slopes for the diet-induced changes, both after 2 and after 8 wks of intervention, were about twice as steep for DHA as for AA. Prima facie this could be taken as evidence for a less efficient incorporation of AA into breast milk TLs compared with DHA, which would be in agreement with most observational studies [143]. However, this interpretation would ignore the considerable differences between the intercepts of the AA and DHA regressions. Moreover, different incorporation efficiencies are not supported by our observation that a comparable daily LCPUFA intake [400 mg AA (high AA group) or 320 mg DHA + 80 mg EPA (DHA + EPA group)] raised the breast milk AA and DHA concentrations after 8 wks by about the same extent (~0.2 g/100 g compared with the control group; see **figure 1A**, high AA vs. control group for AA and **figure 1C**, DHA + EPA vs. control group for DHA). This observation rather suggests that the incorporation efficacies of dietary AA and DHA in breast milk TLs are of the same magnitude. To fully elucidate, however, the efficiency by which dietary AA is incorporated into breast milk TLs, studies with stable isotope-labeled AA are required.

In line with earlier studies [156-158], breast milk total fatty acid concentrations decreased considerably during the course of lactation (**Table 3**). In the control group, AA and the Σn-6 LCPUFAs also declined (**Figures 1A**, **B**). Although these changes were not significant for DHA and the Σn-3 LCPUFAs (**Figures 1C**, **D**), the breast milk AA:DHA and Σn-6:Σn-3 LCPUFA ratio's (1.81 ± 0.37 and 1.77 ± 0.21 at baseline, respectively) remained essentially unchanged. From the 3 experimental groups, it became obvious that additional LCPUFA consumption can substantially alter these ratios. Thus, the extra intake of 400 mg/d DHA + EPA resulted in a reduction of the AA:DHA ratio of 45.8 % within the 8-wks intervention, which brings it within the lower range of the AA:DHA ratio distribution worldwide [143]. In the same period, the Σn-6:Σn-3 LCPUFA ratio declined by 31.3 %. The extra intake of 400 mg/d AA attenuated the decreases of both ratios to 28.6 and 22.1 %, respectively, but did not prevent them. Whether these ratio changes in breast milk composition are of any functional importance, needs to be considered in future studies.

In conclusion, we demonstrated that the consumption by lactating women of additional AA and n-3 LCPUFAs increased the AA and DHA concentrations of their milk TLs. For AA, this effect appeared dose-dependent. Nonetheless, higher amounts of AA than DHA would be required to keep the breast milk AA:DHA ratio constant at habitual values upon DHA supplementation.

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Chapter 7

Prenatal arachidonic acid exposure and selected immune-related variables in childhood

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Abstract

Arachidonic acid (AA) is considered essential in fetal development and some of its metabolites are thought to be important mediators of the immune responses. Therefore, we studied whether prenatal exposure to AA is associated with some immune-related clinical conditions and plasma markers in childhood. In 280 children aged 7 years, atopy, lung function and plasma inflammation markers were measured and their relationships with early AA exposure were studied by linear and logistic regression analyses. AA exposure was deduced from AA concentrations in plasma phospholipids (PLs) of the mothers collected at several time points during pregnancy and at delivery, and in umbilical cord plasma and arterial and venous wall PLs. In unadjusted regression analyses, significant positive associations were observed between maternal AA concentrations at 16 and 32 weeks of pregnancy (proxies for fetal AA exposure) and peak expiratory flow decline after maximal physical exercise and plasma fibrinogen concentrations of their children, respectively. However, after correction for relevant covariables, only trends remained. A significant negative relationship was observed between AA concentrations in cord plasma (reflecting prenatal AA exposure) and the average daily amplitude of peak expiratory flow at rest, which lost significance after appropriate adjustment. Because of these few, weak and inconsistent relationships, a major impact of early-life exposure to AA on atopy, lung function and selected plasma inflammation markers of children at 7 years of age seems unlikely.

Introduction

There is increasing evidence that exposure to nutritional factors, such as n-6 and n-3 polyunsaturated fatty acids (PUFAs), during the perinatal period may influence the development of the immune system and subsequent immune competence [4]. The n-6 and n-3 PUFAs are important structural components of cell membranes and particularly arachidonic acid (AA) is prominently present in immune cell membrane phospholipids (PLs) [159,160]. AA may influence the developing immune system because it is the precursor of prostaglandin E2 (PGE2), which is thought to be an important mediator of immune responses [44,45]. During pregnancy, the maternal essential fatty acid (EFA) status declines and because the EFA status of the neonate is strongly correlated with the EFA status of its mother [26], the AA supply to the developing fetus and its immune system may not always be optimal.

Previous studies investigated the relationship between the maternal intake of n-3 long-chain polyunsaturated fatty acids (LCPUFAs) during pregnancy and immune-related variables of their offspring [161,162]. However, only a few studies considered the relationship between the prenatal n-6 LCPUFA status, especially AA, and immune-related variables, such as atopy later in life. Galli and coworkers observed that babies who developed atopic disease after 1 year of birth had 20-40 % lower levels of AA in their serum cord blood cells in comparison with non-atopic babies [163]. In contrast, Yu et al. found no significant differences in any of the fatty acid levels, measured in umbilical venous blood samples, of babies who did or did not develop allergic diseases during the first 6 years of life [164]. Furthermore, Newson et al. studied the relationship between levels of various n-3 and n-6 series fatty acids, including AA, in erythrocyte PLs of women in late pregnancy and in erythrocyte PLs of the umbilical cord of their children, and the prevalence of wheezing and eczema of the child from birth until 6 months and from age 30-42 months [165]. No significant associations were observed after adjustment for covariables. Thus, the relationship between prenatal AA concentrations and later immune-related variables remains relatively unexplored. Therefore, we investigated in a mother-child cohort the associations between the AA levels of maternal plasma PLs, during early, middle and late pregnancy (as proxies for fetal AA exposure), and several immune-related clinical conditions and inflammation markers of the child at 7 years of age. In addition, we also investigated if these immune-related variables were associated with PL AA concentrations collected in cord plasma and vessel walls of the newborns, all assumed to reflect prenatal AA exposure.

Subjects and methods

Study population and design

The Maastricht Essential Fatty Acid Birth (MEFAB) cohort resulted from observational studies performed during 1990-1997, which investigated the associations between maternal or neonatal EFA status during pregnancy and pregnancy outcome in approximately 1200 pregnant women and most of their infants [1,7]. In 1997, the parents of the 750 singleton infants born between 1990 and 1994 were approached for an extensive follow-up and eventually 305 children participated in these studies between 1997 and 2000 [166,167]. Results of this follow-up were also entered in the MEFAB database, which provided all data for the present study. Children with an unknown gestational age at birth or who were born before 37 weeks gestational age or after 43 weeks of pregnancy were excluded (n = 25). Ultimately, the data of 280 children and their mothers were available for the present statistical analysis. Unadjusted and multivariable-adjusted regression models were applied to explore if maternal or neonatal AA concentrations, measured during pregnancy and/or directly after delivery, are related to childhood immune-related variables.

Blood and tissue sampling and fatty acid measurements

Maternal venous blood samples were collected in EDTA tubes around the 16th, 22nd and 32nd week of pregnancy, and immediately after delivery. Umbilical cord blood and a piece of the umbilical cord were obtained immediately after parturition. About 7 years later, after an overnight fast, venous blood from the children was collected by venepuncture in EDTA-treated evacuated tubes. Plasma was separated from blood cells by centrifugation. Plasma and umbilical tissue samples were stored under nitrogen at -80 °C until analysis. The fatty acid composition of PLs isolated from plasma and cord vein and artery walls was determined by capillary gas-liquid chromatography as described elsewhere [26,68]. Fatty acids are expressed as relative values (% by weight of total identified PL-associated fatty acids, % wt/wt).

Explanatory variables

Four explanatory neonatal variables were taken to reflect prenatal AA exposure, i.e. the relative AA concentrations of PLs isolated from umbilical plasma and from the walls of the cord vein and artery, and the difference between these latter two concentrations, which we considered a proxy for fetal AA consumption [75]. The AA concentrations of maternal plasma PLs at approximately 16, 22 and 32 weeks of gestation and at delivery were applied as four additional explanatory variables. These variables are taken to reflect the AA status of the

fetus during gestation, since the maternal and neonatal EFA statuses are strongly correlated [26].

Dependent variables

The following immune-related variables were modelled as dependent variables in the regression analyses: presence of atopic clinical conditions, peak expiratory flow (PEF) outcomes and plasma concentrations of a number of inflammation markers (all described later).

Atopy assessment. For the atopy assessment of the children, parents completed an atopy questionnaire, based on the 'Maastricht Atopy List' [168] and the well-validated [169,170] 'International Study of Asthma and Allergies in Childhood (ISAAC)' questionnaire [171]. This questionnaire included queries about typical atopy-related symptoms, categorized per atopic organ [172], such as 'Has your child ever had allergic complaints such as eczema, hay fever, and/or food allergy?', 'Has your child ever had asthma or asthmatic bronchitis?', and 'Has your child ever had wheezing or whistling in the chest'. In addition, the following question about allergy tests was included: 'Has your child ever undergone an allergy test like a skin-prick test, radioallergosorbent test (RAST), elimination test or otherwise? If yes, what was the test result?'. From these questionnaires, the presence of atopy was evaluated by a paediatric pulmonologist (JJEH), and scored 'yes' or 'no' when, respectively, more than one or none of the above-mentioned clinical conditions were conclusively manifest. Children whose diagnosis was inconclusive (scoring only one of the above-mentioned clinical conditions) were excluded from analysis to prevent bias introduced by misclassification.

Peak flow measurements. Asthma is a chronic inflammatory disorder of the airways in which many different cell types play a role. In susceptible individuals this inflammation causes symptoms which are usually associated with widespread but variable airflow obstruction, that is often reversible either spontaneously or with treatment, and causes an associated increase in airway responsiveness to a variety of stimuli [173]. PEF measurements are often used in everyday clinical and epidemiological environments for measuring the severity in airflow obstruction of asthma, to characterize the clinical trial population and to describe the response to treatment [174]. In addition, investigators of previous studies observed that in children with asthmatic symptoms atopy was associated with a greater within-day and between-day variation in PEF [175,176]. To assess pulmonary function in the present study, PEF was measured using a Mini-Wright peak flow meter. Measurements were carried out at rest (at home) and before and after maximal physical exercise (at the laboratory), both after instructions of the research team. Under the supervision of the parents, PEF measurements at rest were performed for 2 weeks, five times per day during the morning (between 07.00 and 09.00 hours)

and five times per day during the evening (between 19.00 and 21.00 hours). Each day, the highest PEF value in the morning and the highest value in the evening were recorded. To increase the reliability of the measurements, these values were only accepted if at least two other PEF values (of that series of five measurements) fell within a range of 10 % of that highest value. If this was not the case, this procedure was repeated with the next highest PEF value, etc. until two PEF values fell within the 10 % range of that next highest PEF value, otherwise a missing value was generated. The average highest PEF value at rest in the morning (PEF morning) was calculated for the 2-week measurement period. In addition, the PEF daily amplitude was calculated as the difference between the highest morning and highest evening PEF value and expressed as a percentage of the mean [177]. The average daily PEF amplitude (PEF amplitude) was calculated over the 2-week measurement period. To measure PEF decline after exercise provocation (PEF exercise), PEF measurements were performed at the laboratory three times before, and three times at 2, 5, 10 and 15 minutes after reaching maximal exercise with the Bruce treadmill test [178,179], of which the highest PEF value was recorded each time. PEF decline after exercise provocation was calculated as: {(PEF highest value before exercise - PEF lowest value after maximal exercise) / PEF highest value before exercise} x 100 % [180].

Plasma inflammation markers. The following factors are involved in different processes of the inflammatory response and were therefore chosen as dependent variables: fibrinogen (g/L) [181], C-reactive protein (CRP; mg/L) [182], leptin (µg/L) [183] and von Willebrand factor (vWF) concentrations [184]. In addition, total leucocyte counts (x 10^9/L blood) were measured, as well as the absolute and relative amounts of lymphocytes, granulocytes and monocytes (% of total leucocytes) [185].

Fibrinogen was assayed by the Clauss method [186] and CRP was measured with an in-house ELISA using polyclonal antibodies as catching and tagging antibodies labelled with horseradish peroxidase (DAKO, Glostrup, Denmark). Plasma leptin concentrations were measured with a commercial human leptin radioimmunoassay kit (Linco Research, St Charles, MO, USA) according to the manufacturer's instructions. The relative plasma content of vWF was measured by the Cejka method [187]. The inter-assay and intra-assay variation coefficients were 3.3 and 3.6 % (fibrinogen), 6.5 and 3.2 % (CRP), 8.2 and 6.0 % (leptin), 5.1 and 5.7 % (vWF). Total leucocyte counts and relative granulocyte, lymphocyte, and monocyte counts were determined on a Coulter Counter (Coulter® GEN.S; Beckmann Coulter Inc. Fullerton, CA) following the manufacturer’s instructions.

Covariables

The following variables were considered potential confounders: socio-economic status (SES) [188], parity (none, one or ≥ two children) at study entry [7,189], maternal smoking during pregnancy and anyone smoking at home during the 7-year follow-up period (both measured as total number of cigarettes/1000) [190], breast-feeding (number of months) [191], maternal age at study entry (years) [192], infant sex [193], gestational age (weeks) [7,26], parent ethnicity (Caucasian or not) [193], birth season (divided into quarters) [194], season of follow-up measurements (divided into quarters) [195], day-care attendance (total number of days/100) [196], parental history of atopy (none, one of the parents, or both) assessed at follow-up [191] and weight gain during first year of life corrected for infant sex and age (SD scores) [197]. The relative dihomo-γ-linolenic acid (DGLA), EPA and DHA concentrations in umbilical cord plasma and vein and artery wall PLs, and in maternal plasma PLs, as well as the relative AA concentration of plasma PLs of the children at follow-up were also selected as potential covariables [43]. Exact information on SES was not available. Therefore, parental SES was measured by proxy, using 'income' as an SES indicator, based on the parental postal code at the time of delivery (Geomarktprofiel; Wegener DM, The Netherlands). This information was classified in five groups ranging from 1 (twice or more modal income) to 5 (minimum income); SES values in the categories unknown (0) and diverse (6) were omitted and, thus, reported as missing values. Children who never received breast milk were classified as formula-fed and the remaining children as breast-fed. The following covariables were considered confounders in the models with PEF-related outcomes only: endurance time (time required until maximal exercise was reached, abstracted from the Bruce treadmill test) [178], site of first PEF measurement (at the laboratory with extensive instructions by the research team or at home, with written instructions only) and children's height and weight at follow-up (age 7 years) [198].

Data evaluation and statistical analysis

All data are presented as medians and interquartile ranges (IQR), unless otherwise mentioned. Unadjusted and multivariable-adjusted linear and logistic regressions were performed to test the associations between maternal and neonatal AA concentrations and the immune-related variables. Before this, data distributions of the dependent variables were checked by means of the Shapiro-Wilk test; in case of skewness transformation was applied (natural log, square root, square or 1/square) to optimize the data distribution towards normal. When transformation improved but did not normalize the distribution, linear regression analyses were still performed, but results were accepted only if the residuals were normally distributed. If this was not the case, the variables were dichotomized and logistic regression analyses were performed. In the

regression analyses with maternal AA levels this appeared the case for absolute monocyte counts and leptin concentrations. Therefore, these variables were analyzed as dichotomous variables (≥ median vs. < median). The same was done for fibrinogen and leptin concentrations in relation to neonatal AA concentrations. The vWF values were biphasically distributed with the point of overlap situated at 68 %. Therefore, this variable was analyzed as a dichotomous variable also (≥ 68 vs. < 68 %). Because the PEF exercise variable had a non-parametric distribution, this variable was also dichotomized. Children without an asthmatic condition usually have a smaller PEF decline (< 15 %) after exercise [173], because they will develop less bronchoconstriction compared with their asthmatic counterparts. Therefore, this value was selected as the threshold for dichotomisation (≥ 15 vs. < 15 %).

Values of normally distributed variables (either before or after transformation) were considered outliers and removed from the dataset if they were more than four standard deviations (SD) away from the mean. Values of not normally distributed variables, even after optimal transformation, were considered outliers and were removed from the dataset if their values were more than three IQR below or above the median.

Insufficient complete cases were available to allow inclusion of all the above-mentioned covariables in the analyses. Therefore, for each of the fifteen dependent variables and each covariable, bivariable regression analyses were performed which included one dependent variable, one explanatory variable and one covariable. These analyses were used to determine which of the covariables contributed to the relationships between dependent and explanatory variables, either as a significant predictor ($p < 0.050$) or as a confounder (if its removal caused the B-value to change at least 10 %, *and* 20 % or more of the standard error of this B-value) [104]. Only covariables that appeared predictors or confounders were included in the various multivariable-adjusted analyses. For practical reasons, bivariable analyses were only done for AA concentrations in maternal plasma PLs at 32 weeks gestation and for AA concentrations in cord artery wall PLs. The selected covariables were subsequently included in all models with corresponding maternal or neonatal AA variables. For all unadjusted and multivariable-adjusted regression analyses the same complete dataset was used. Data points suspected of being overly influential were checked by calculation of their Cook's distances and removed if their values were > 1. Such influential data points were observed in four multivariable-adjusted regression models, but exclusion of these data points did not alter the final results.

Atopy and the three pulmonary function variables were regarded as the dependent variables of primary interest. Relationship studies with these variables were, therefore, considered the primary analyses. All other relationships were regarded of secondary interest. The significance level for the primary analyses was set at a threshold of $p < 0.050$, with $p < 0.100$ indicating a

non-significant trend. Secondary relationships were considered significant at $p < 0.010$, to correct for multiple comparisons, and $p < 0.050$ indicated a non-significant trend.

SPSS 11.5 for Windows (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses.

Results

An overview of the parental and infant characteristics screened as covariables are shown in **tables 1** and **2**. The immune-related parameters of the children measured at 7 years of age are presented in **table 3**. The relative concentrations of the maternal and neonatal fatty acids of interest is given in **table 4**. In **tables 5** and **6**, results of the regression analyses are only shown for combinations of dependent and explanatory variables with significant or trend contributions of AA to either the unadjusted or multivariable-adjusted models. Full results are available on request.

Table 1. Maternal and child characteristics used as continuous covariables

Characteristics	n	Median (25^{th} - 75^{th} percentile)
Maternal age at study entry (years)	280	29.5 (26.9 - 32.4)
Gestational age at birth (weeks)	280	40.1 (39.3 - 41.0)
Infant weight gain during first year of life (SD)	247	1.0 (0.3 - 1.7)
Height at 7-year follow-up (cm)	275	127.0 (123.2 - 130.4)
Weight at 7-year follow-up (kg)	275	24.5 (22.2 - 27.6)
Day-care attendance during 7-year follow-up (total days/100)	272	0.08 (0.08 - 0.12)
Breast-feeding (months)	278	0.00 (0.00 - 3.00)
Maternal smoking during pregnancy (total cigarettes/1000)	280	0.00 (0.00 - 0.54)
Anyone smoking at home during 7-year follow-up (total cigarettes/1000)	276	5.29 (0.00 - 25.6)
Endurance time at 7-year follow-up (min)*	248	10.5 (10.0 - 11.2)

* Time required to reach maximal exercise, calculated from the Bruce test results [178] and considered a measure of physical fitness.

Table 2. Frequency and code for relevant discrete parental and infant covariables

	n	%
Parental covariables		
Parents' SES grade (income)		
High (1)	4	1.4
Above modal (2)	23	8.2
Modal (3)	44	15.7
Below modal (4)	74	26.4
Low (5)	31	11.1
Missing	104	37.1
Total	280	100
Parental atopy, positive		
None	148	52.9
Father	44	15.7
Mother	64	22.9
Both	19	6.8
Missing	5	1.8
Total	280	100
Parity		
0	193	68.9
1	68	24.3
≥2	19	6.8
Missing	0	0
Total	280	100
Parents' ethnicity		
Caucasian (0)	270	97.1
All other (1)	8	2.9
Missing	0	0
Total	280	100
Infant covariables		
Infant sex		
Male (0)	152	54.3
Female (1)	128	45.7
Missing	0	0
Total	280	100
Birth season quartiles*		
1	71	25.4
2	49	17.5
3	71	25.4
4	88	31.4
Missing	1	0.4
Total	280	100
Season of measurement quartiles*		
1	97	34.6
2	75	26.8
3	61	21.8
4	47	16.8
Missing	0	0
Total	280	100
Site of first PEF measurement		
At laboratory (0)	106	37.9
At home (1)	152	54.3
Missing	22	7.9
Total	280	100

SES = socio-economic status; PEF = peak expiratory flow. * 1 = January - March, 2 = April - June, 3 = July - September, 4 = October - December.

Table 3. Immune-related variables measured in children at follow-up at age 7 years

Variable	Unit	n	Median (25^{th} - 75^{th} percentile)*
	no	183 (65.4 %)	n.a.
Atopy	uncertain	45 (16.1 %)	n.a.
	yes	52 (18.6 %)	n.a
PEF morning	L/min	257	214.58 (193.96 - 242.50)
PEF amplitude	%	255	7.89 (5.80 - 10.28)
PEF exercise	%	257	5.26 (0.00 - 10.53)
Leucocytes	$\times 10^9$/L	243	6.60 (5.80 - 7.70)
Monocytes	$\times 10^9$/L	243	0.40 (0.30 - 0.50)
Granulocytes	$\times 10^9$/L	245	3.60 (2.80 - 4.60)
Lymphocytes	$\times 10^9$/L	246	2.50 (2.10 - 3.00)
Monocytes	%	245	6.20 (4.65 - 8.00)
Granulocytes	%	244	53.95 (47.83 - 62.50)
Lymphocytes	%	246	38.95 (32.00 - 44.43)
CRP	mg/L	232	0.19 (0.07 - 0.58)
Leptin	µg/L	245	2.85 (2.07 - 3.93)
Fibrinogen	g/L	233	2.60 (2.40 - 2.80)
vWF	%	233	81.00 (71.00 - 84.00)

n.a. = not applicable; PEF = peak expiratory flow; PEF morning = average PEF in the morning; PEF amplitude = average PEF daily amplitude; PEF exercise = PEF decline after exercise provocation; CRP= C-reactive protein; vWF= von Willebrand factor. * For all variables non-transformed values are given.

Table 4. Relative concentrations (% wt/wt) of arachidonic acid (AA, 20:4n-6), docosahexaenoic acid (DHA, 22:6n-3), dihomo-γ-linolenic acid (DGLA, 20:3n-6) and eicosapentaenoic acid (EPA, 20:5n-3) in neonatal and maternal plasma PLs

Matrix	n	AA	DHA	DGLA	EPA
Umbilical plasma	280*	16.89 (15.84 - 17.89)	6.07 (5.17 - 7.15)	5.18 (4.69 - 5.72)	0.21 (0.17 - 0.27)
Umbilical artery wall	186	13.69 (12.28 - 15.36)	5.22 (4.56 - 5.92)	1.27 (1.09 - 1.45)	n.d
Umbilical vein wall	185	18.19 (17.05 - 19.28)	5.00 (4.42 - 5.61)	1.86 (1.66 - 2.16)	n.d.
Umbilical vein - artery wall difference	183	4.48 (3.13 - 5.52)	-0.23 (-0.63 - 0.14)	0.61 (0.39 - 0.76)	n.d.
Plasma at 7 years of age	246	9.21 (8.29 - 10.03)	2.68 (2.33 - 3.21)	2.94 (2.58 - 3.29)	0.47 (0.37 - 0.59)
Maternal plasma, 16 weeks	256	9.55 (8.52 - 10.45)	4.03 (3.43 - 4.57)	3.00 (2.68 - 3.52)	0.45 (0.34 - 0.57)
Maternal plasma, 22 weeks	237	8.53 (7.66 - 9.43)	4.09 (3.66 - 4.66)	3.30 (2.94 - 3.73)	0.36 (0.27 - 0.45)
Maternal plasma, 32 weeks	250	8.11 (7.38 - 8.72)	3.94 (3.48 - 4.45)	3.38 (2.97-– 3.71)	0.30 (0.23 - 0.39)
Maternal plasma, partus	252	8.29 (7.41 - 9.35)	3.78 (3.35 - 4.32)	3.40 (3.06 - 3.85)	0.30 (0.22 - 0.39)

PLs = phospholipids; n.d = not detectable. Values are given as median (25th - 75th percentile). * n = 273 for EPA.

Associations between maternal arachidonic acid status and childhood immune-related clinical parameters

A negative trend was observed for the unadjusted association between the natural log-transformed PEF amplitude and maternal AA levels at the 22nd week of pregnancy (R^2 = 0.016, p = 0.069), but this trend was no longer present in the multivariable-adjusted analysis (**Table 5**) .

A significant positive association and a positive trend were observed for the unadjusted relationships between PEF exercise values and the AA levels in maternal plasma at 16 weeks of pregnancy (p = 0.033) and directly after delivery (p = 0.097), respectively. In these unadjusted models, differences in AA concentrations explained 4.5 and 2.8 % of the variance in PEF exercise, respectively. After adjustment of both models for relevant covariables, only a positive trend remained for the first relationship (p = 0.059), in which AA explained 3.5 % of the variation in PEF exercise.

For all other combinations of maternal AA concentrations and immune-related clinical parameters no significant associations or trends were found.

Associations between the maternal arachidonic acid status and childhood immune-related plasma variables

The only significant relationship observed in the unadjusted analyses was a positive association between the AA levels at week 32 of pregnancy and fibrinogen concentrations in children's plasma at follow-up (linear regression analysis after quadratic transformation of fibrinogen concentrations, p = 0.006) (**Table 5**). After adjustment for the child's AA concentration at follow-up, the association lost significance (p = 0.014, which is higher than 0.010 that was set for the secondary analyses) and differences in maternal AA concentrations explained 2.9 % of the variance in fibrinogen levels.

Negative trends were observed for the unadjusted associations of the relative and absolute monocyte counts with AA concentrations at the 16th week of pregnancy. The models explained 2.1 and 4.5 % of the variance in relative (p = 0.030) and absolute (p = 0.034) monocyte counts, respectively. These trends persisted after adjustment for the appropriate covariables, which resulted in models in which the AA concentrations explained 1.8 % (relative monocyte values, p = 0.039) and 4.4 % (absolute monocyte counts, p = 0.030) of the monocyte variance.

All other regression analyses revealed no significant associations or trends.

Table 5. Unadjusted and multivariable-adjusted linear (LIN) and logistic (LOG) regression analyses of the relationships between plasma phospholipid (PL) arachidonic acid (AA) concentrations of mothers sampled during pregnancy and at delivery, and immune-related variables measured in their children at 7 years of age[a]

Pregnancy week	Dependent variables	LIN or LOG	n	Unadjusted analysis results						Multivariable-adjusted analysis results[b]						
				R^2	B	β or OR	p	CI (95 %)		R^2	B	β or OR	r^2	p	CI (95 %)	
								L	U						L	U
Primary analyses																
22	PEF amplitude (%, ln[h])	LIN	207	.016	-0.04	-0.13	.069	-0.09	0.00	.040	-0.03[c]	-0.09	.008	.204	-0.08	0.02
16	PEF exercise (≥ 15 % decline)	LOG	212	.045	0.34	1.40	*.033*	1.03	1.90	.077	0.33[d]	1.38	.035	.059	0.99	1.94
Delivery	PEF exercise (≥ 15 % decline)	LOG	208	.028	0.27	1.31	.097	0.95	1.80	.050	0.20[d]	1.22	.014	.251	0.87	1.73
Secondary analyses																
32	Fibrinogen (g/L, square[i])	LIN	208	.036	0.28	0.19	*.006*	0.08	0.47	.044	0.25[e]	0.17	.029	.014	0.05	0.45
16	Monocytes (%)	LIN	225	.021	-0.24	-0.14	.030	-0.45	-0.02	.077	-0.22[f]	-0.13	.018	.039	-0.43	-0.01
16	Monocytes (≥ 0·40 $\times 10^9$/L)	LOG	139	.045	-0.27	0.77	.034	0.60	0.98	.155	-0.30[g]	0.74	.044	.030	0.56	0.97

[a] R^2 = coefficient of determination for linear regression, Nagelkerke R square for logistic regression; B = unstandardized regression coefficient; β = the standardized regression coefficient of B for linear regression (LIN), which is the number of outcome-standard deviations (SD) that the outcome will change as a result of one predictor-SD change in the predictor; OR = Odds Ratio for logistic regression (LOG); p = p-value of AA contribution; CI (95 %) = confidence interval of B for linear regression and of OR for logistic regression, both set at 95 %; L = lower bound of CI; U = Upper bound of CI; r^2 = square of the semi-partial correlation coefficient of AA. PEF = peak expiratory flow. *Italic numbers* refer to a significant relationship ($p < 0.050$ for primary analyses; $p < 0.010$ for explorative analyses), a non-significant trend was indicated as $0.050 \leq p < 0.100$ for primary analyses and as $0.010 \leq p \leq 0.050$ for secondary analyses. [b] The total model p-values of the (final) multivariable-adjusted analyses were between 0.000 and 0.178. [c] covariables: site of first PEF measurement and maternal plasma PL DHA concentrations. [d] covariables: DHA concentrations in maternal plasma PL and AA concentration in children's plasma PLs at follow-up. [e] covariable: AA concentration in children's plasma PLs at follow-up. [f] covariable: infant sex. [g] covariables: infant sex, DHA concentrations in maternal plasma PLs and parent's socioeconomic status. [h] natural log transformation. [i] square transformation.

Associations between neonatal arachidonic acid status and childhood immune-related clinical parameters

Unadjusted regression analysis showed a significant negative association between natural log-transformed PEF amplitude values and AA concentrations in neonatal plasma PLs (R^2 = 0.026, p = 0.020) (**Table 6**). However, after adjustment for relevant covariables, this association was reduced and no longer significant.

A positive trend was observed for the unadjusted relationship between PEF exercise and the difference in AA concentrations between the walls of cord arteries and cord vein (p = 0.087), which explained 4.6 % of the variance in PEF exercise. This positive trend disappeared, however, after adjustment for the appropriate covariables.

No significant associations or trends were observed for the other combinations of neonatal AA concentrations and clinical immune-related variables.

Associations between neonatal arachidonic acid status and childhood immune-related plasma variables

In unadjusted regression analyses, only a positive trend was observed for the relationship between absolute lymphocyte counts (square root-transformed) of the children at follow up, and their plasma PL AA concentrations at birth (R^2 = 0.018, p = 0.038), but this trend did not survive after adjustment for DGLA and DHA concentrations in neonatal plasma PLs (**Table 6**).

In multivariable-adjusted analyses, a negative trend was found for the relationship between the natural log-transformed plasma CRP levels of the children at follow up and the AA concentrations measured in the PLs of their cord artery walls (p = 0.019). In this model, AA explained 3.7 % of the CRP variance.

A positive trend (p = 0.049) was observed for the multivariable-adjusted relationship between children's natural log-transformed absolute leucocyte counts at age 7years and the AA levels of their cord vein wall PLs. In this relationship, AA explained 2.3 % of the variance in leucocyte count.

No other important associations or trends were noted.

Table 6. Unadjusted and multivariable-adjusted linear (LIN) and logistic (LOG) regression analyses of the relationships between phospholipid (PL) arachidonic acid (AA) concentrations in several umbilical domains of neonates, and their immune-related variables measured at 7 years of age[a]

Umbilical domain	Dependent variables	LIN or LOG	n	Unadjusted analysis results						Multivariable-adjusted analysis results[b]						
				R^2	B	β or OR	p	CI (95 %)		R^2	B	β or OR	r^2	p	CI (95 %)	
								L	U						L	U
Primary analyses																
Plasma vein-artery difference	PEF amplitude (%, ln)[c]	LIN	212	.026	-0.04	-0.16	*.020*	-0.07	-0.01	.106	-0.03[e]	-0.11	.008	.171	-0.06	0.01
	PEF exercise (≥ 15 % decline)	LOG	143	.046	0.26	1.30	.087	0.96	1.76	.130	0.18[f]	1.19	.010	.396	0.79	1.79
Secondary analyses																
Plasma	Lymphocytes ($\times 10^9$/L, square root)[d]	LIN	246	.018	0.02	0.13	.038	0.00	0.03	.028	0.02[g]	0.12	.011	.105	-0.00	0.03
Artery wall	CRP (mg/L, ln)[c]	LIN	137	.020	-0.10	-0.14	.101	-0.21	0.02	.153	-0.19[h]	-0.28	.037	.019	-0.35	-0.03
Vein wall	Leucocytes ($\times 10^9$/L, ln)[c]	LIN	162	.023	0.02	0.15	.054	0.00	0.04	.107	0.02[i]	0.17	.023	.049	0.00	0.04

[a] R^2 = coefficient of determination for linear regression, Nagelkerke R square for logistic regression; B = unstandardized regression coefficient; β = the standardized regression coefficient of B for linear regression (LIN), which is the number of outcome-standard deviations (SD) that the outcome will change as a result of one predictor-SD change in the predictor; OR = Odds Ratio for logistic regression (LOG); p = p-value of AA contribution; CI (95 %) = confidence interval of B for linear regression and of OR for logistic regression, both set at 95 %; L = lower bound of CI; U = Upper bound of CI; r^2 = square of the semi-partial correlation coefficient of AA. PEF = peak expiratory flow. *Italic numbers* refer to a significant relationship ($p < 0.050$ for primary analyses; $p < 0.010$ for explorative analyses), a non-significant trend was indicated as $0.050 \leq p < 0.100$ for primary analyses and as $0.010 \leq p \leq 0.050$ for secondary analyses. [b] The total model p-values of the (final) multivariable-adjusted analyses were between 0.006 and 0.093. [c] natural log transformation. [d] square root transformation. [e] covariables: site of first PEF measurement, maternal age, DGLA and DHA concentrations in neonatal plasma PLs, endurance time, parity and infant sex. [f] covariables: umbilical vein-artery differences of DGLA and DHA concentrations, first year weight gain and height at follow-up. [g] covariables: DGLA and DHA concentrations in neonatal plasma PLs. [h] covariables: parity, maternal smoking during pregnancy, anyone smoking at home during follow-up period, first year weight gain, DGLA and DHA concentrations in umbilical artery wall PLs and AA concentration in plasma PLs of children at follow-up. [i] covariables: birth season, DGLA and DHA concentrations in umbilical vein wall PLs and infant sex.

Discussion

In the unadjusted primary analyses of this prospective mother-child cohort, negative associations were observed between the average PEF daily amplitude (PEF amplitude) of 7-year-old children and the AA concentrations in plasma PLs of their mothers at 22 weeks gestation (reflecting fetal exposure to AA) and in umbilical plasma PLs (reflecting prenatal AA exposure). On the other hand, positive associations were observed between the PEF decline after maximal exercise (PEF exercise) and maternal AA concentrations at 16 weeks of pregnancy and directly after delivery, respectively. These positive associations are in concordance with the positive non-significant trend between PEF exercise and neonatal vein-artery AA differences, which we consider a proxy of fetal AA consumption. For these associations and trends, variations in AA levels explained no more than 0.8 to 3.5 % of the variability of these immune-associated variables, strongly suggesting that the prenatal AA status of an individual hardly contributes to these immune-related aspects at 7 years of age. This is also supported by the finding that the associations are not only functionally inconsistent but contrasting as well. Since values for PEF amplitude and/or PEF exercise are usually increased in subjects with an asthmatic condition [(173)], the negative associations we observed with AA exposure imply a beneficial effect of AA on lung function, if relations are causal. In contrast, the positive relationships between AA exposure and PEF exercise indicate a potentially adverse effect of AA. Finally, in the multivariable-adjusted analyses most significant results and trends disappeared.

Concerning the secondary analyses with the immune-related plasma markers, one significant positive association was shown between maternal AA concentrations at 32 weeks of pregnancy and plasma fibrinogen concentrations of the children at 7 years of age. For some other associations only trends were revealed but, like the primary analyses, no more than about 4 % of the functional variability was explained by differences in AA concentration. Moreover, observed associations and trends were functionally inconsistent again and therefore a major influence of early AA exposure on these immune-related plasma markers at age 7 years seems unlikely.

Relationships between early-life exposure to n-3 and n-6 fatty acids and immune-related clinical conditions measured during childhood have been previously studied [(199-202)]. However, as far as we know, only a few studies investigated the associations between prenatal n-6 fatty acid exposure and various indicators of immune function in childhood. Newson and co-workers did not report any significant association between relative AA concentrations in erythrocyte PLs of umbilical cord blood and maternal blood collected in a period between 20 weeks of pregnancy and delivery and the prevalence of wheezing or eczema up to 42 months of age after adjustment for confounders [(165)]. Furthermore, Yu et al. also observed no difference in the relative AA levels of

umbilical cord blood PLs of children who did or did not develop allergic disease during the first 6 years of life [164]. Both findings are in line with our observation that plasma AA concentrations before and around birth are not strongly associated with atopic disease in later childhood. Since these previously mentioned studies used shorter periods between fatty acid exposure measurements and assessment of immune-related variables, it can probably be excluded that the interval period of 7 years in the present study was too long to explain the non-significant results.

Galli and co-workers selected fifty-seven neonates at high risk for developing atopy and reported that all thirteen newborns who developed atopic disease during the first 12 months of follow-up had significantly (20-40 %) lower AA levels in their cord blood PLs at birth as compared with their non-atopic counterparts [163]. However, no correction was made for potential confounders which may have biased their results. In addition, Sausenthaler et al. suggested that intake of n-6 fatty acid-rich foods, such as margarines and vegetable oils, during the last 4 weeks of pregnancy was associated with an increased risk of allergic diseases in children at the age of 2 years [162]. Although these foods contain a high content of linoleic acid (LA), the increased risk could also be related to one of the many other components of these products. Furthermore, it is known from a previous study that a higher maternal intake of LA does not relate to higher AA concentrations in maternal and umbilical plasma PLs [203].

A strong aspect of the present study is that maternal and neonatal fatty acid concentrations were obtained repeatedly during pregnancy and at parturition, respectively. Since maternal and neonatal LCPUFA concentrations are strongly correlated [7], this enabled us to investigate the associations between the child's immune-related variables at age 7 years and the AA exposure from the second trimester of pregnancy on.

Although we tested all relevant variables included in the database for their confounding or predicting potentials, residual confounding cannot be excluded, which is a general shortcoming of observational studies. Due to the absence of relevant data, we were unable to correct our analyses for differences in the postnatal AA consumption of the children, which may have contributed to the inter-individual differences in immune outcome variables at age 7 years. However, we did correct for the plasma PL AA concentration at age 7 years, which can be considered a proxy for the postnatal AA intake, since there is a positive relationship between dietary AA intake and plasma PL AA concentration [204,205].

The use of a parent questionnaire to assess the atopic state of the children is clearly inferior to a full clinical evaluation. However, in large-scale observational studies, questionnaires are often the only practical option to gather clinical information. It should be added that the questionnaire we used was based on the ISAAC questionnaire [171], which has been properly validated [169,170], showing sensitivity and specificity of at least 77 and 81 %, respectively.

Furthermore, as mentioned before all questionnaires were 'scored' by an experienced clinician (JJEH). Therefore, although not optimal, we consider our atopy-assessment method adequate for the present observational study. Finally, we cannot exclude response bias in this cohort study, since informed consent for the 7-year follow-up was obtained for only 300 out of the 750 eligible mother-child pairs.

In the present study, no clear associations were observed between atopy at age 7 years and prenatal AA exposure as reflected by maternal AA concentrations during pregnancy and at delivery and neonatal AA values at birth. In theory, this may result from too-narrow ranges in AA concentrations and/or a too-low atopy incidence in our study population in relation to our sample size. However, the AA concentrations measured in our study population compare very well with maternal [96,206] and neonatal [206,207] values observed in other studies, whereas the atopy incidence in our population is relatively high as compared with the atopy prevalence reported in a world-wide study [208]. Since the Odds Ratio's for the atopy risk as a function of the AA concentrations in the various domains are all close to 1 (0.79-1.11 for the eight domains studied) and their 95 % confidence intervals are rather tight around 1 (the widest interval being 0.66-1.33), we consider the power of the present study sufficient for a reliable conclusion. Comparable conclusions can be drawn for most other immune-associated variables. This supports the absence of relevant associations between the perinatal AA availability and selected aspects of the immune status at the age of 7 years.

In the present study, the same cases were used in the unadjusted as well as the multivariable-adjusted regression models, enabling us to check whether confounding was present and in which direction confounding influenced the model. In general, results were comparable when unadjusted regression analyses were performed with the maximum number of cases available (data not shown).

Finally, in the present study outliers were removed before statistical analyses were applied. Usually an outlier should only be omitted from the statistical analyses if there is a biological explanation as to why this value is inappropriate. Repeating the statistical analyses with the outliers included did not fundamentally alter the results: non-significant results did not become significant and vice versa. So, the final results and the conclusions of this study did not change when the outliers were added in the analyses.

In conclusion, we investigated whether normally occurring differences in prenatal AA availability could be of importance for the later presence of atopy, lung function and plasma inflammation markers. From the results obtained, such an influence seems rather unlikely since physiological differences in prenatal exposure to AA show few, weak and inconsistent associations with differences in several immune-related clinical conditions and plasma markers at 7 years of age. Genetic predisposition and *in utero* exposure to other factors are

possibly more important determinants of these variables than prenatal AA concentrations. However, because of the limitations of the present observational study (mentioned above), the involvement of early AA status in the development of the immunological system cannot be excluded.

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Chapter 8

General discussion

Introduction

During pregnancy and the first years of life many developmental milestones are reached and it is crucial that high quality nutrition can be supplied to the fetus and thereafter to the infant to ensure that all essential nutrients are provided. Of these nutrients, the essential long-chain polyunsaturated fatty acids (LCPUFAs) docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6) are thought to be of critical importance for brain development and fetal growth, respectively [2,3]. Mothers receive the LCPUFAs mainly from their diet or synthesize them from their respective precursors [95]. To obtain these fatty acids, fetuses depend on their mothers, as is indicated by the positive correlation between the maternal and neonatal LCPUFA status at birth [6-8]. However, pregnancy is thought to be associated with a decrease in the maternal LCPUFA status, which may have consequences for fetal development.

Two studies described in this thesis were therefore carried out to assess fetal brain function on the basis of habituation measurements and to explore if these specific brain functions, i.e. fetal learning and memory, are associated with the early fetal availability of essential fatty acids (EFAs) and their LCPUFAs, collectively named the essential PUFAs (ePUFAs) [1].

Since some LCPUFAs are thought to be important factors for fetal growth, two studies were conducted in a mother-child pregnancy-birth cohort to explore whether some selected birth dimensions are associated with fetal exposure to LCPUFAs and *trans* fatty acids, reflected by maternal and neonatal LCPUFA and *trans* fatty acid concentrations measured during pregnancy and/or directly after birth.

In lactating women a decline of the relative DHA levels is found when lactation progresses [27], which may not be optimal for infant development. This decline can probably be prevented by maternal DHA supplementation, but elevated n-3 LCPUFA consumption often coincides with a decrease in the AA concentrations of an individual. In pregnant and lactating women, however, this may not be desirable, since AA is considered essential for fetal and infant development [31,32]. Therefore, a study was carried out to investigate whether it is possible to prevent a DHA decline without lowering the AA levels by supplementing lactating women with a combination of n-3 LCPUFAs and AA.

Finally, since studies revealed that the relative maternal AA concentrations decrease during pregnancy [26], the neonatal AA status may not be optimal. AA is the precursor of an important immune response mediator, prostaglandin E2 (PGE2) [44,45], and therefore we investigated, in the above mentioned mother-child cohort, if prenatal AA exposure is associated with some immune-related clinical conditions measured at 7 years of age.

This chapter will provide the main conclusions of the studies described in this thesis, in relation to various methodological considerations, and will give some suggestions for further research.

Fetal habituation and the ePUFA status

Habituation is a decrease leading to cessation of a behavioural response that occurs when an initially novel stimulus is repeatedly presented [35]. It is often considered to represent a form of learning and probably requires an intact and functioning central nervous system (CNS) [35]. In this thesis, fetal habituation was used to assess two forms of brain function, i.e. fetal memory and learning, and to relate these functions to the early ePUFA status of the fetus.

In these habituation tests, every 30 seconds a 1-second stimulus was applied to the maternal abdomen above the fetal legs. Movements of the fetus within 1 second after application of the stimulus were considered a positive response. Disappearance of the response for four consecutive stimuli was taken to demonstrate habituation. The habituation rate (HR) was defined as the number of consecutive stimuli applied before habituation was established. The initial habituation tests (HR-A) at 30, 32, 34, 36 or 38 weeks gestational age (GA), considered as a measure of the fetal learning capacity at the moment of the habituation measurements, were repeated 10 minutes later. For groups 30-36, both measurements were replicated during a second session at GA 38. The different time periods between habituation tests were used to assess fetal short-term memory (10-minutes intervals) and long-term memory (2-8 weeks intervals).

In each group, with the exception of group 32, we observed a significant decline between the initial and repeated habituation tests in both sessions, which indicates the presence of a short-term (10 minutes) memory. This short-term memory appeared GA-independent, just like fetal learning. In addition, there were some indications that fetuses of 34 weeks GA can already store information and retrieve it 4 weeks later, see **chapter 2**. However, the presence of this long-term memory measured in fetuses of 38 weeks GA was not very strong, because the habituation data of these fetuses at 38 weeks GA were not significantly different from the data of fetuses of the same age who were never tested before.

One suggestion for further research on this long-term memory is related to the concern that in the present study the longest interval was tested in the youngest fetuses. Therefore, it would be better in further research to start the first session with fetuses of the same age. Since it is observed that fetuses between 37 and 40 weeks GA have a memory of at least 24 hours [48], whereas an other study did not observe significant differences when 34-35 week old fetuses were re-tested 7 days after the first session [52], it might be an option to start memory-intervals from 1 day on and build these up to 1 week, 2 weeks, etc.

As we already mentioned in **chapter 2**, a limitation of our study was that we did not include a test necessary to distinguish habituation from receptor adaptation or effector fatigue. Habituation can be distinguished from adaptation

or fatigue by the recovery of a habituated response on presentation of a new stimulus (dishabituation) and faster habituation upon re-presentation of the original stimulus [33,35]. It needs to be mentioned that in a study of Leader et al. it was observed that dishabituation could only be observed in up to 79 % of the normal fetuses tested [63]. Furthermore, it appears difficult to apply a novel stimulus that is sufficiently different from the original stimulus, because any new stimulus that is too similar to the original stimulus will not elicit a recovery of the response [34,63]. Therefore, it seems useful to develop other methods than dishabituation as described here to indicate when habituation has occurred.

We used the described habituation technique to investigate if fetal learning and memory were associated with the early ePUFA status, see **chapter 3**. The results suggest that, if associations are causal, fetal short-term (10 minutes) memory measured before 38 weeks GA may be better the lower the ePUFA status of the fetus, as reflected by higher MA and MA+DHMA levels. Furthermore, fetal long-term memory would be better the lower the n-3 LCPUFA status, as indicated by higher ObA concentrations. These findings are not in line with the current opinion that ePUFAs, especially AA and DHA, are important for brain development and function [37,38]. Because we only observed a few trends between the habituation-related fetal brain functions and the early ePUFA status, we concluded that physiological differences in the availability of these fatty acids may probably not determine the differences in these primitive brain functions during the third trimester of fetal development. The question then is whether the power of our study was sufficient to come to reliable conclusions. Since the 95 % confidence intervals of the fetal learning and memory variables as a function of the ePUFA status include '1' and are rather wide, we conclude that our study can not reliably demonstrate the absence of relevant associations between these forms of brain function and the ePUFA status because the power is too low. For the observed trends we can also conclude that the power is too low to make reliable statements about the magnitude of the associations. As far as we know, this is the first time that these forms of fetal brain functions, measured by the habituation technique, are linked to the ePUFA status of these fetuses. Therefore, it was not possible to make power calculations in advance because the size of the effects was not known.

LCPUFAs and fetal growth

In our study, maternal plasma phospholipid (PL) DHA concentrations, especially when measured early in pregnancy, were significantly and *positively* associated with birth weight and head circumference, whereas maternal AA and dihomo-γ-linolenic acid (DGLA, 20:3n-6) concentrations in late pregnancy were *negatively* related to birth weight and birth length. In **chapter 4**, these results are extensively discussed in relation to other peer-reviewed articles.

Our findings suggest that, if results are causal, maternal AA and DGLA later in pregnancy might be involved in fetal growth limitation and that maternal DHA contents may programme fetal growth in a positive way, especially early in pregnancy. This latter could imply that DHA supplementation only before or during early pregnancy may significantly affect birth outcomes. However, in a recently published study of Drouillet et al., it was investigated whether fetal growth is associated with maternal fatty acid intake *before* and during the last 3 months of pregnancy of French women included in the EDEN mother-child cohort study [(209)]. Of these pregnant women, 74.3 % had a body mass index (BMI) < 25 kg/m^2 before pregnancy. No significant associations were observed between birth dimensions and the proportion of n-3 fatty acids in total PUFA intake (measured by a food frequency questionnaire (FFQ)) before and at the end of pregnancy. These results were observed in multivariable-adjusted regression analyses, which were corrected for BMI among others. Nevertheless, a significant role for DHA early in pregnancy cannot be precluded since n-3 fatty acids were not measured separately but assessed as an n-3 fatty acid package, including DHA, docosapentaenoic acid (DPA, 22:5n-3) and eicosapentaenoic acid (EPA, 20:5n-3). For the n-6 fatty acids also no significant associations were found in the same population. In addition, no specification was given in the article about the included fatty acids accounting for the n-6 fatty acids. Although the used FFQ is closely related to a validated FFQ used in the Fleurbaix-Laventie Ville Santé Study [(210,211)], additional not-validated questions were added for a more specific assessment of the intake of foods rich in n-3 fatty acids and seafood. This could mean that the FFQ was not sensitive enough to measure the fatty acid status.

In **chapter 5**, we reported that DHA and AA concentrations measured in various umbilical domains and considered to reflect fetal LCPUFA availability during late gestation were mainly negatively related to birth weight and birth length. Our results seem to preclude their role as growth factors *per se*, but the negative relationships observed may result from a limited maternal-fetal LCPUFA transfer capacity.

Arachidonic acid and immune function

In **chapter 7**, we investigated whether prenatal exposure to AA is associated with some immune-related variables in childhood. From our results it can be concluded that such an influence seems rather unlikely since we only found few, weak and inconsistent associations between prenatal exposure to AA and several immune-related clinical conditions and plasma markers at 7 years of age.

For the multivariable-adjusted regression analyses of this study we selected relevant covariables based on bivariable regression analyses. These analyses

were used to determine which of the covariables contributed to the relationships between dependent and explanatory variables, either as a significant predictor or as a confounder. One of these variables was 'AA concentration in children's plasma PLs at follow-up', now abbreviated as 'AA at follow-up'. We included this variable because we wanted to correct for differences in the *postnatal* AA consumption of the children, which may have contributed to the inter-individual differences in immune outcome variables at age 7. However, these data were not available and therefore we corrected for the plasma PL AA concentrations at 7 years of age, which can be considered a proxy for the postnatal AA intake, since there is a positive relationship between dietary AA intake and plasma PL AA concentrations [204,205]. However, significant tracking in this cohort has recently been observed for the plasma PL AA levels between age 7 (=X) and birth ($Y = 6.31 + 0.17X$, $r = 0.231$, $n = 249$, $p = 0.0002$; G. Hornstra, to be published). Therefore, it is probably not appropriate to correct for AA levels at follow-up in our analyses because this may reduce the fetal exposure range of AA, the explanatory variable in the regression analyses and, thereby, the power of the study. As a consequence, we considered it necessary to remove this variable from all regression analyses in which it was involved either as a predictor or as a confounder, *viz*.:

1) Neonatal AA *vs*. CRP
2) Maternal AA *vs*. PEF exercise
3) Maternal AA *vs*. fibrinogen
4) Maternal AA *vs*. CRP

The outcomes indicate that removal of the variable 'AA at follow-up', results in stronger and significant *positive* associations between the peak expiratory flow decline after maximal exercise (PEF exercise) and maternal AA concentrations at 16 weeks of pregnancy (reflecting fetal exposure to AA) and between fibrinogen and maternal AA concentrations at 32 weeks GA. If these associations are causal, these results indicate a stronger negative effect of AA on these immune-related variables, than described in **chapter 7**. Although, stronger associations are observed still not more as 4.5 % of the variability of these immune-associated variables is explained by variations in maternal AA levels. Regarding the neonatal AA levels, the trend between AA concentrations measured in the PL of the cord artery walls and CRP was lost after removal of the 'AA at follow-up' variable.

Methodological considerations

MEFAB cohort and study design

Three studies described in this thesis can be identified as observational historical cohort studies. In this kind of research, populations are defined based on data collected at a given time in the past. These three studies used data of the Maastricht Essential Fatty Acid Birth (MEFAB) cohort. This cohort originated from observational studies performed during 1990-1997, which investigated the associations between the maternal and neonatal essential fatty acid statuses during pregnancy and pregnancy outcome in approximately 1200 pregnant women and most of their infants [1,7]. Between 1997 and 2000, a follow-up study was initiated and from the 750 eligible children, born between 1990 and 1994, around 300 children participated at 7 years of age [166,167]. Results of this follow-up were also entered in the MEFAB database, which provided all data for the studies described in **chapters 4, 5 and 7**.

Given the growing recognition of the importance of the life course approach for the risk assessment of chronic diseases, birth cohort studies are becoming increasingly important. The MEFAB cohort contains invaluable information about mothers and their children from pregnancy onwards. This can be used to disentangle essential questions about the prenatal LCPUFA status, which is related to the maternal diet, and its relation in child development and the state of health at a later age. If significant associations are observed, and if results are causal, then there is the possibility to contribute to optimal development, prevent illnesses and promote health of these children through proper maternal diet adjustments. Furthermore, because of the large array of variables, this cohort proofed also suitable for observational studies with respect to body weight [212,213], physical activity [214] and insulin resistance [215]. In addition, the MEFAB database has been used to study problem behaviour of children in relation to their early LCPUFA exposure [216].

Biological relationships, for example between the intake of an essential nutrient and functions dependent on that nutrient, are probably not linear, but plateau at intakes above need [217]. However, in most of our statistical analyses linear regression models were used, which imply that as the value of X (the explanatory variable) increases so the value of Y increases by an amount equal to the regression coefficient. Also for our data, relations are most probably monotonous, which means that they either increase or decrease over the whole range of X. However, it was not advisable to check if non-linear models were more appropriate because the associations were, in overall, not very strong. This makes it hardly feasible to explore more complicated relations than linear, since each association would then have to be modelled by one or more extra variables. The same argument holds against modelling the association using dummy variables for three or more categories of X. Moreover, such modelling

would often fail since the number of variables in the model would get too large for the number of cases in the analysis.

A limitation of observational studies is that residual confounding cannot be ruled out because associations can be influenced by effects of unmeasured variables or by imperfectly measured relevant variables. The data for these current studies, however, were retrieved from a database containing a relatively large number of maternal and neonatal variables, which reduces the chance to observe an association based on a not-included variable. Furthermore, data from the original and the follow-up studies in 1997-2000 were embedded in the same research line, in which hypotheses were closely related to the ones in the present thesis. This latter decreases the probability of less perfectly measured variables.

Absolute versus relative fatty acid concentrations

Fatty acid data can be expressed in absolute amounts (mg/L erythrocyte suspension/plasma or mg/g dry tissue) as well as relative concentrations (% of total fatty acids). Both expression methods have their own limitations. When relative concentrations are used, the problem is created that an increase in one fatty acid automatically results in decreases in all other relative fatty acid proportions, because the sum of all known fatty acids is 100 %. This methodological problem is hard to overcome, but in our studies we chose to express the fatty acids as relative amounts to take into account potential differences in the total lipid pool sizes of mothers and their fetuses. Furthermore, because pregnancy is associated with hyperlipidemia, pool sizes in maternal blood are changing considerably over time during pregnancy. Moreover, because metabolic interactions between fatty acids occur, it is the amount of a given fatty acid relative to the other fatty acids present that determines the rate of this reaction in the specific environment. Therefore, from a metabolic perspective it is important to report fatty acids as relative values. In addition, measurement errors of the analytical procedure between different laboratories are in general smaller when fatty acids are expressed as relative amounts.

Using maternal and neonatal fatty acid concentrations as a proxy for fetal fatty acid exposure

Since maternal and neonatal EFA and LCPUFA plasma PL contents are positively correlated at birth [(218)], it can be suggested that maternal fatty acids during pregnancy reflect the prenatal fatty acid exposure. Van Houwelingen and colleagues collected human fetal tissue after elective abortions in the first trimester and determined the fatty acid composition of the fetal tissue. At the same time of the abortion, a maternal blood sample was obtained to measure

the fatty acid composition in maternal erythrocytes PLs [128]. For the sum of n-6 fatty acids, LA, the sum of n-3 fatty acids and DHA significant positive correlations were observed between maternal and fetal fatty acid levels.

In another study, the fatty acid composition of umbilical plasma samples obtained by fetal blood sampling during ongoing pregnancies in the second and third trimester, was compared to the fatty acid composition of umbilical cord blood samples, collected immediately after birth of infants of the same gestational age [124]. It was observed that fetuses do not possess a different EFA status than infants directly after birth at a comparable gestational age. This suggests that the EFA status measured at birth appears a reasonably adequate reflection of the fetal EFA status. Moreover, most relative maternal plasma PL fatty acid values, collected at the same time, were highly correlated with the fetal fatty acid concentrations, confirming that maternal fatty acid concentrations can be used as a proxy for fetal fatty acid exposure.

Practical implications of our findings

The studies described in this thesis have various practical implications, which are briefly pointed out below.

- Measurement of fetal learning and memory could lead to a better understanding of the normal development of the fetal CNS. However, fetal habituation is probably not a suitable method to investigate whether the maternal diet can influence these primitive forms of fetal brain function (**Chapter 3**).

- If the observed association between maternal DHA concentrations and birth weight in **chapter 4** is causal and if a low birth weight is a predictor for a poor prognosis later in life, than it is prudent to promote maternal DHA intake before pregnancy. Just like it is nowadays advised to take folic acid to prevent neural tube defects.

- In the study described in **chapter 4**, negative associations were observed between maternal AA concentrations and birth weight and birth length. Based on these results, it seems prudent for now not to increase the AA levels of pregnant women, to reduce the change of a probably negative influence of AA on birth weight.

- *Trans* fatty acids may compromise fetal development by lowering the fetal LCPUFA status. In our studies, effects of maternal or neonatal 18:1*t*, the main industrially produced *trans* unsaturated fatty acid present in the diet, were either small or non-existing (**Chapters 4** and **5**). This might be partly

explained by the rather low 18:1*t* contents in the plasma phospholipids of our study population. However, for populations with a high habitual *trans* consumption it seems necessary to further investigate the potential role of *trans* fatty acids on birth outcome in these populations. These populations often include humans of underprivileged groups in developed countries and humans in developing countries.

- Several studies indicated that an increase in the consumption of n-3 LCPUFAs often causes a concomitant decrease of circulating n-6 LCPUFA concentrations and of AA in particular. However, we observed in **chapter 6** that consumption of extra DHA + EPA by lactating women increased the sum of n-3 LCPUFA and DHA concentrations in their breast milk total lipids, whereas no measurable effect was observed for breast milk AA or the sum of n-6 LCPUFAs. Thus, if it is felt necessary during the lactation period to improve the maternal LCPUFA status, and especially the DHA status, than it is possible to consume more DHA without affecting the AA levels in breast milk.

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Summary & Samenvatting

Summary

Essential fatty acids and their longer-chain more unsaturated derivatives, the LCPUFAs, are collectively named 'essential PUFAs'. These fatty acids belong, according to the position of the first double bond, to the n-6 or n-3 fatty acid family. Especially arachidonic acid (AA, 20:4n-6) and docosahexaenoic acid (DHA, 22:6n-3) are LCPUFAs which are thought to be important for fetal growth and brain development, respectively. Therefore, the availability of these fatty acids for the fetus needs to be adequate. To obtain these fatty acids, fetuses depend on their mother's essential PUFA consumption and metabolism as well as on the placental transfer of these fatty acids. Since pregnancy is associated with a decrease in the biochemical LCPUFA status of the mother, the fetal and neonatal LCPUFA status may not be optimal and this may affect fetal and neonatal development.

In order to investigate if fetal brain functions are associated with the early essential PUFA status, a method was used which measures fetal habituation. From these measurements several aspects of fetal brain function, such as fetal learning and memory, can be deducted.

In **chapter 2**, we used fetal habituation to explore from which gestational age fetal learning and memory can be established, how long fetal memory lasts and whether fetal learning and memory depend on fetal age. Ninety-three pregnant women were recruited to assess fetal learning and memory between 30-38 weeks gestational age, by recurrent habituation tests. Our results showed that fetal learning appeared to be gestational age independent. Furthermore, it was observed that fetuses have a short-term (10 minutes) memory from a gestational age of at least 30 weeks onwards which also appeared to be independent of the fetal age, when measured for the first time. In addition, we presented some evidence that 34 week old fetuses are able to store information and retrieve it 4 weeks later.

In **chapter 3**, these brain functions, i.e. fetal learning, fetal short-term memory and long-term memory, were related to the early essential PUFA status of 72 fetuses. The essential PUFA status was deduced from concentrations of AA and DHA, from their essential fatty acid dietary precursors [linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3)], from Mead acid (MA, 20:3n-9), dihomo-Mead acid (DHMA, 22:3n-9) and MA+DHMA and from Osbond acid (ObA, 22:5n-6). The levels of these fatty acids were measured in the phospholipids of the umbilical artery walls. The presence of relatively high contents of MA, DHMA and MA+DHMA indicate a poor essential PUFA status and these fatty acids are therefore defined as the general essential PUFA status markers. Large contents of ObA point to a poor n-3 LCPUFA status and as a result ObA is indicated as an n-3 LCPUFA status marker. For AA, DHA and LA no significant associations were observed with fetal learning or memory.

On the other hand, positive trends were found for the relationships between fetal short-term memory and concentrations of the general essential PUFA status markers MA and MA+DHMA. Furthermore, a positive trend was also observed for the association between fetal long-term memory and levels of ObA, a marker of the n-3 LCPUFA status. If causal, these relationships indicate that fetal short-term memory measured before 38 weeks gestational age may be better, the lower the essential PUFA status of the fetus, as reflected by higher MA and MA+DHMA concentrations. Likewise, fetal long-term memory would be better the lower the n-3 LCPUFA status, as indicated by higher ObA concentrations. These interpretations are in striking contrast with current opinions. Moreover, these associations are rather weak and therefore we suggested that physiological differences in essential PUFA availability may probably not determine the differences in these primitive brain functions during the third trimester of fetal development.

Since there are indications that low birth weight is associated with negative health outcomes later in life, it is sensible to optimize fetal growth.

In **chapter 4**, the data of 782 mother-infant pairs of the Maastricht Essential Fatty Acid Birth (MEFAB) cohort were used to study the associations between birth weight, birth length or head circumference and the relative contents of DHA, AA, dihomo-γ-linolenic acid (DGLA, 20:3n-6) and *trans*-octadecenoic acid (18:1*t*) in maternal plasma phospholipids sampled during early, middle and late pregnancies, and at delivery. With multiple linear regression analyses, significant *positive* associations were observed between maternal DHA levels (especially early in pregnancy) and birth weight and head circumference. Significant *negative* associations were found between maternal AA levels, measured at late pregnancy and directly after delivery, and birth weight and birth length. Also significant *negative* associations were observed between maternal DGLA concentrations, measured at delivery, and birth weight and birth length. No significant associations were found for maternal 18:1*t* contents. We concluded that, if these relationships are causal, maternal DHA during early pregnancy may programme fetal growth in a positive way. Maternal AA and DGLA in late pregnancy might be involved in fetal growth limitation.

In **chapter 5**, we investigated with linear regression analyses if birth weight, birth length or head circumference are associated with the prenatal exposure to relative concentrations of DHA, AA, DGLA and 18:1*t* measured in phospholipids of the walls of umbilical arteries and veins, of umbilical cord plasma and of erythrocytes. For this study the data of up to 700 infant-mother pairs from the MEFAB cohort were used. After optimal adjustment, a significant negative association was observed between birth weight and umbilical plasma DHA concentrations. Significant negative associations were also found for the relationships between birth weight and AA levels measured in umbilical plasma, and in arterial and venous vessel walls. For birth length, a significant negative

association was observed with arterial vessel wall AA concentrations only. A significant negative association was also found for the relationship between 18:1*t* in cord erythrocytes and birth weight. For DGLA no significant associations were observed. These results seem to preclude a role of DHA and AA as growth factors *per se*. Their negative relationships with birth dimensions may result from a limited maternal-fetal LCPUFA transfer capacity. Potential effects of 18:1*t* and DGLA on birth dimensions are probably small or non-existing.

In lactating women a decline of the relative DHA levels is found in plasma phospholipids when lactation progresses. These levels become even lower than those of non-lactating mothers and also lower than those before conception. Since DHA seems to be important for brain and retina development and function of young infants, this may jeopardize the optimal developmental potentials of breastfeeding. Recommended increased consumption of n-3 LCPUFAs may lower the AA status of lactating women, which may be unfavorable to their infants also, since AA is considered essential for fetal and infant development.

In **chapter 6**, we therefore studied the effects of n-3 LCPUFA consumption, with or without AA, on DHA and AA levels in breast milk and erythrocytes of lactating women. A total of 52 healthy pregnant women were included in the study. During the lactation period, these women were randomly allocated to four different groups, a control group receiving no additional LCPUFAs, a low or a high AA group in combination with n-3 LCPUFAs, or a group who received n-3 LCPUFAs alone. Blood and breast milk samples were collected to measure the relative concentrations of AA, DHA and sums of n-6 and n-3 LCPUFAs in erythrocyte phospholipids and in milk total lipids. The combined consumption of AA and n-3 LCPUFAs caused dose-dependent elevations of AA and total n-6 LCPUFA concentrations in milk total lipids and did not significantly affect the DHA and total n-3 LCPUFA increases caused by the n-3 LCPUFAs in the supplement. Treatment with n-3 LCPUFAs only did not significantly affect breast milk AA and total n-6 LCPUFA concentrations. AA and DHA concentrations in milk total lipids and their changes were strongly and positively correlated with their corresponding values in erythrocyte phospholipids. We concluded that the consumption by lactating women of additional AA and n-3 LCPUFAs increased the AA and DHA concentrations of their milk total lipids. For AA, this effect appeared dose-dependent, which is a novel finding not reported before.

AA is the precursor of prostaglandin E2, which is thought to be an important mediator of immune responses. Since the AA status becomes reduced during pregnancy, the neonatal AA status may not be optimal which may have consequences for the developing immune system.

In **chapter 7**, we explored if prenatal exposure to AA as represented by maternal or neonatal AA concentrations, measured during pregnancy and/or directly after delivery, is related to several immune-related variables. For this investigation the data of 280 children and their mothers were retrieved from the MEFAB database. Linear and logistic regression analyses were used to study if the early exposure to AA is related to lung function, presence of atopy and inflammation markers in the 7-year-old children. In the unadjusted regression analyses several significant associations were observed. However, after correction for relevant covariables, only trends remained. Since most observed associations and trends were functionally inconsistent, a major influence of early AA exposure on these immune-related clinical conditions and plasma markers at 7 years of age seems unlikely.

In **chapter 8**, the experimental results are extensively discussed. The conclusions and their practical implications are briefly pointed out below.

- Measurement of fetal learning and memory could lead to a better understanding of the normal development of the fetal central nervous system. However, fetal habituation is probably not a suitable method to investigate whether the maternal diet can influence these primitive forms of fetal brain function.

- If the observed association between maternal DHA concentrations and birth weight is causal and if a low birth weight is a predictor for a poor prognosis later in life, than it is prudent to promote maternal DHA intake before pregnancy.

- Since negative associations were observed between maternal AA concentrations and some birth dimensions, it seems prudent for now not to increase the AA levels of pregnant women.

- In our studies, effects of maternal *trans* fatty acid consumption on fetal growth appeared either small or non-existing. This might be partly explained by the rather low 18:1*t* contents in the plasma phospholipids of our study population. However, for populations with a high habitual trans consumption it seems necessary to further investigate the potential role of *trans* fatty acids on birth outcome.

- Finally, if it is felt necessary during the lactation period to improve the maternal LCPUFA status, and especially the DHA status, than it is possible to consume more DHA without affecting the AA levels in breast milk.

Samenvatting

Essentiële vetzuren en hun langere-keten hoger-onverzadigde afgeleiden, de LCPUFAs, worden samen ook wel ‘essentiële PUFAs’ genoemd. Deze vetzuren behoren, volgens de positie van de eerste dubbele binding, tot de n-6 of n-3 vetzuur familie. Met name arachidonzuur (AA, 20:4n-6) en docosahexaeenzuur (DHA, 22:6n-3) zijn LCPUFAs waarvan wordt gedacht dat ze belangrijk zijn voor, respectievelijk, de foetale groei en hersenontwikkeling. Daarom is het belangrijk dat de foetus voldoende van deze vetzuren tot zijn beschikking heeft. Om deze vetzuren te verkrijgen zijn de foetussen afhankelijk van hun moeders essentiële PUFA consumptie en metabolisme als ook van de overdracht van deze vetzuren via de placenta. Aangezien zwangerschap geassocieerd is met een daling in de biochemische LCPUFA status van de moeder is de foetale en neonatale LCPUFA status wellicht niet optimaal en dit kan gevolgen hebben voor de foetale en neonatale ontwikkeling.

Om te onderzoeken of foetale hersenfuncties geassocieerd zijn met de vroege essentiële PUFA status maakten we gebruik van een methode die foetale habituatie meet. Van deze meetresultaten kunnen meerdere aspecten van foetale hersenfunctie, zoals foetaal leervermogen en geheugen, worden afgeleid.

In **hoofdstuk 2** hebben we foetale habituatie gebruikt om te onderzoeken vanaf welke zwangerschapsduur foetaal leervermogen en geheugen kunnen worden vastgesteld, hoe lang foetaal geheugen voortduurt en of foetaal leervermogen en geheugen afhankelijk zijn van de foetale leeftijd. Drieënnegentig zwangere vrouwen werden geworven om met behulp van herhaalde habituatie testen het foetale leervermogen en geheugen, tussen week 30 en 38 van de zwangerschap, te meten. Onze resultaten toonden aan dat het foetale leervermogen onafhankelijk blijkt te zijn van de foetale leeftijd. Verder werd geconstateerd dat foetussen in ieder geval vanaf week 30 van de zwangerschap een korte termijn geheugen hebben van 10 minuten, dat onafhankelijk blijkt te zijn van de foetale leeftijd wanneer het voor de eerste keer wordt gemeten. Onze resultaten suggereerden ook dat 34 weken oude foetussen in staat zijn om informatie op te slaan en die 4 weken later terug te halen.

In **hoofdstuk 3** werden deze hersenfuncties, te weten het foetale leervermogen, het foetale korte termijn en lange termijn geheugen, gerelateerd aan de vroege essentiële PUFA status van 72 foetussen. De foetale essentiële PUFA status werd afgeleid van de concentraties van AA en DHA, van hun essentiële vetzuur voorlopers [linolzuur (LA, 18:2n-6) en alfa-linoleenzuur (ALA, 18:3n-3)], van Meadzuur (MA, 20:3n-9), dihomo-Meadzuur (DHMA, 22:3n-9) en MA+DHMA en van Osbondzuur (ObA, 22:5n-6). De concentraties van deze vetzuren werden gemeten in de fosfolipiden van de wanden van de

navelstrengarteriën. De aanwezigheid van relatief grote hoeveelheden MA, DHMA en MA+DHMA wijst in de richting van een ongunstige essentiële PUFA status en deze vetzuren worden daarom ook wel aangeduid als de algemene essentiële PUFA status markers. Grote hoeveelheden ObA duiden daarentegen op een ongunstige n-3 LCPUFA status en daarom wordt ObA ook wel gezien als een n-3 LCPUFA status marker. Voor AA, DHA en LA werden er geen significante associaties waargenomen met het foetale leervermogen en geheugen. Wel werden er positieve trends gevonden voor de relaties tussen het foetale korte termijn geheugen en concentraties van de algemene essentiële PUFA status markers MA en MA+DHMA. Verder werd er ook een positieve trend gevonden voor de associatie tussen het foetale lange termijn geheugen en de n-3 LCPUFA marker, ObA. Indien deze relaties causaal zijn dan duiden ze aan dat het foetale korte termijn geheugen, gemeten vóór week 38 van de zwangerschap, beter kan zijn naarmate de essentiële PUFA status van de foetus lager is, zoals weerspiegeld wordt door hogere MA en MA+DHMA niveaus. Ook zou het foetale lange termijn geheugen beter zijn naarmate de n-3 LCPUFA status lager is, zoals geïndiceerd door de hogere ObA concentraties. Deze verklaringen druisen in tegen de heersende opvattingen. Bovendien zijn deze associaties nogal zwak en daarom suggereerden we uiteindelijk dat fysiologische verschillen in de essentiële PUFA beschikbaarheid waarschijnlijk niet de verschillen in deze primitieve hersenfuncties kunnen bepalen gedurende het derde trimester van de foetale ontwikkeling.

Aangezien er aanwijzingen zijn dat een laag geboortegewicht geassocieerd is met negatieve gezondheidsuitkomsten, later in het leven, is het verstandig om de foetale groei bij geboorte te optimaliseren.

In **hoofdstuk 4** werden de gegevens van 782 moeder-kind paren van het 'Maastricht Essential Fatty Acid Birth' (MEFAB) cohort gebruikt om de relaties te bestuderen tussen geboortegewicht, geboortelengte of hoofdomtrek en de relatieve concentraties van DHA, AA, dihomo-γ-linoleenzuur (DGLA, 20:3n-6) en *trans*-octadeceenzuur (18:1*t*). Deze vetzuren zijn aanwezig in de fosfolipiden van moederlijk plasma verzameld tijdens de vroege, middelste en late periode van de zwangerschap en direct na de bevalling. Met meervoudige lineaire regressie analyses werden significante *positieve* associaties waargenomen tussen moederlijke DHA concentraties (met name vroeg in de zwangerschap) en geboortegewicht en hoofdomtrek. Significante *negatieve* associaties werden gevonden tussen moederlijke AA concentraties, gemeten tijdens de late zwangerschap en direct na de bevalling, en geboortegewicht en geboortelengte. Ook werden er significante *negatieve* associaties gevonden tussen moederlijke DGLA concentraties, gemeten direct na de bevalling, en geboortegewicht en geboortelengte. Er werden geen significante associaties waargenomen voor moederlijke 18:1*t* concentraties. We concludeerden dat, indien deze relaties causaal zijn, moederlijk DHA gedurende de vroege

zwangerschap foetale groei op een positieve manier kan programmeren. Moederlijk AA en DGLA zijn tijdens de late zwangerschap wellicht betrokken bij foetale groeibeperking.

In **hoofdstuk 5**, onderzochten we met lineaire regressie analyses of geboortegewicht, geboortelengte of hoofdomtrek geassocieerd zijn met de prenatale blootstelling aan relatieve concentraties van DHA, AA, DGLA en 18:1*t*. Deze vetzuren zijn gemeten in fosfolipiden geïsoleerd uit de wanden van de navelstrengarteriën en -venen en uit navelstrengplasma en erytrocyten. Voor deze studie werden de gegevens van 700 moeder-kind paren van het MEFAB cohort gebruikt. Na optimale correctie werd een significante negatieve associatie waargenomen tussen geboortegewicht en DHA concentraties gemeten in navelstrengplasma. Significante negatieve associaties werden ook gevonden voor de relaties tussen geboortegewicht en AA concentraties gemeten in navelstrengplasma en in de wanden van de navelstrengarteriën en -venen. Voor geboortelengte werd alleen een significante negatieve associatie gevonden met AA concentraties uit de wand van de navelstrengarterie. Een significante negatieve associatie werd ook waargenomen tussen 18:1*t* in navelstreng erytrocyten en geboortegewicht. Voor DGLA werden er geen significante associaties gevonden. Deze resultaten lijken een rol voor DHA en AA als groeifactoren uit te sluiten. Hun negatieve relaties met geboorte-uitkomsten kunnen voortvloeien uit een beperkte LCPUFA overdrachts-capaciteit tussen de moeder en de foetus. Potentiële effecten van 18:1*t* en DGLA op geboorte-uitkomsten zijn waarschijnlijk klein of bestaan niet.

Bij vrouwen die borstvoeding geven is in plasma fosfolipiden een afname gevonden van de relatieve DHA concentraties. Deze concentraties zijn zelfs lager dan bij moeders die geen borstvoeding geven. Wanneer de borstvoeding voortduurt worden de DHA concentraties zelfs lager dan de niveaus gemeten vóór de zwangerschap. Aangezien DHA belangrijk lijkt te zijn voor de ontwikkeling en functie van de hersenen en retina kan dit wellicht de optimale ontwikkelingsmogelijkheden van borstvoeding in gevaar brengen. Als de consumptie van n-3 LCPUFAs toeneemt, zoals wordt aanbevolen, dan kan de AA status in vrouwen die borstvoeding geven dalen. Dit is wellicht ongunstig voor hun kinderen aangezien AA wordt geacht essentieel te zijn voor de ontwikkeling van de foetus en het kind.

In **hoofdstuk 6** hebben we daarom de effecten onderzocht van n-3 LCPUFA consumptie, met of zonder AA, op DHA en AA concentraties in moedermelk en erytrocyten van vrouwen die borstvoeding geven. In totaal werden er 52 gezonde zwangere vrouwen in de studie geïncludeerd. Deze vrouwen werden tijdens de borstvoedingsperiode willekeurig ingedeeld in 4 verschillende groepen, een controle groep die geen additionele LCPUFAs kreeg, een lage of een hoge AA groep in combinatie met n-3 LCPUFAs, of een groep die alleen n-3 LCPUFAs kreeg. Bloed en moedermelk monsters werden

verzameld om de relatieve concentraties van AA, DHA en de som van n-6 en n-3 LCPUFAs te meten in de fosfolipiden van erytrocyten en in totaal melkvet. De gecombineerde consumptie van AA en n-3 LCPUFAs zorgde voor dosis-afhankelijke verhogingen van AA en totale n-6 LCPUFA concentraties in totaal melkvet en had geen significante invloed op de DHA en totale n-3 LCPUFA toenames, veroorzaakt door de n-3 LCPUFAs in het supplement. Toediening van n-3 LCPUFAs alleen had geen significante invloed op moedermelk AA en totale n-6 LCPUFA concentraties. AA en DHA concentraties in totaal melkvet en hun veranderingen waren sterk en positief gecorreleerd met hun corresponderende waarden in erytrocyt fosfolipiden. Uit de resultaten hebben we geconcludeerd dat, bij vrouwen die borstvoeding geven, de consumptie van extra AA en n-3 LCPUFAs ervoor zorgt dat de AA en DHA concentraties van hun totaal melkvet worden verhoogt. Voor AA blijkt dit effect dosis-afhankelijk te zijn. Dit is een nieuwe bevinding die niet eerder gerapporteerd is.

AA is de voorloper van prostaglandine E2 waarvan gedacht wordt dat het een belangrijke mediator is van immunologische processen. Aangezien de AA status tijdens de zwangerschap daalt is de neonatale AA status wellicht niet optimaal. Dit kan misschien consequenties hebben voor het in ontwikkeling zijnde immuunsysteem.

In **hoofdstuk 7** onderzochten we of prenatale blootstelling aan AA, afgeleid van moederlijke of neonatale AA concentraties, gemeten gedurende de zwangerschap en/of direct na de bevallig, is gerelateerd aan meerdere immuun-gerelateerde variabelen. Voor deze studie werden de gegevens van 280 kinderen en hun moeders gehaald uit de MEFAB database. Lineaire en logistische regressie analyses werden gebruikt om na te gaan of de vroege blootstelling aan AA gerelateerd is aan de longfunctie van 7-jarige kinderen. Bij dezelfde kinderen werd ook gekeken naar de relatie tussen AA en de aanwezigheid van atopische aandoeningen. Als laatste werd er nog gekeken naar de relatie tussen AA en ontstekingsmarkers in plasma. In de niet gecorrigeerde regressie analyses werden meerdere significante associaties gevonden. Maar na correctie voor de relevante covariabelen bleven alleen trends over. Aangezien de meeste associaties en trends functioneel gezien inconsistent waren, lijkt een belangrijke invloed van vroege AA blootstelling op deze immuun-gerelateerde klinische condities en ontstekingsmarkers op 7 jarige leeftijd onwaarschijnlijk.

In **hoofdstuk 8** zijn de experimentele resultaten uitgebreid bediscussieerd. De conclusies en hun praktische toepassingen staan hieronder puntsgewijs beschreven.

- Meting van het foetale leervermogen en geheugen kan bijdragen aan een beter inzicht in de normale ontwikkeling van het foetale centraal

zenuwstelsel. Maar foetale habituatie is waarschijnlijk niet geschikt als basis voor een methode om te onderzoeken of de voeding van de moeder deze primitieve vormen van foetale hersenfunctie kan beïnvloeden.

- Als de gevonden associatie tussen moederlijke DHA concentraties en het geboortegewicht van het kind causaal is en als een laag geboortegewicht een voorspeller is voor een slechte prognose later in het leven, dan is het verstandig om vóór de zwangerschap een toename van de DHA consumptie aan te bevelen.

- Aangezien negatieve associaties werden waargenomen tussen moederlijke AA concentraties en sommige geboorte-uitkomsten, lijkt het verstanding om geen maatregelen te nemen die de AA status van zwangere vrouwen zouden kunnen verhogen.

- In onze studies bleken effecten van moederlijke *trans*vetzuur consumptie op de foetale groei klein of niet aanwezig te zijn. Dit kan waarschijnlijk voornamelijk verklaard worden door de behoorlijk lage 18:1*t* concentraties in de plasma fosfolipiden van onze studiepopulatie. Voor populaties met een hoge *trans* consumptie lijkt het echter nodig om verder onderzoek te verrichten naar de mogelijke rol van *trans*-vetzuren op geboorte-uitkomsten.

- Ten slotte, als het nodig wordt geacht om tijdens de borstvoedingsperiode de moederlijke LCPUFA status te verbeteren, en dan voornamelijk de DHA status, dan is het mogelijk om meer DHA te consumeren zonder dat het de AA concentraties in moedermelk beïnvloedt.

Abbreviations

Abbreviations

18:1*t*	*trans* isomers of octadecenoic acid
AA	arachidonic acid (20:4n-6)
AdrA	adrenic acid (22:4n-6)
ALA	α-linolenic acid (18:3n-3)
ANCOVA	analysis of covariance
ANOVA	analysis of variance
β	standardized regression coefficient of B
B	unstandardized regression coefficient
BL	birth length
BMI	body mass index
BW	birth weight
C	carbon
CH_3	methyl
CI	confidence interval
CNS	central nervous system
COOH	carboxyl
CRP	C-reactive protein
CV	coefficients of variation
DGLA	dihomo-γ-linolenic acid (20:3n-6)
DHA	docosahexaenoic acid (22:6n-3)
DHMA	dihomo-Mead acid (22:3n-9)
DPA	docosapentaenoic acid (22:5n-3)
EDTA	ethylenediaminetetraacetic acid
EFA(s)	essential fatty acid(s)
ELISA	Enzyme-Linked Immuno Sorbent Assay
EPA	eicosapentaenoic acid (20:5n-3)
ePUFA(s)	essential polyunsaturated fatty acid(s)
FFQ	food frequency questionnaire
GA	gestational age
HC	head circumference
HR	habituation rate
IQR	interquartile ranges
ISAAC	International Study of Asthma and Allergies in Childhood

LA	linoleic acid (18:2n-6)
LCPUFA(s)	long-chain polyunsaturated fatty acid(s)
LIN	linear regression
ln	natural log
LOG	logistic regression
LTM	long-term memory
MA	Mead acid (20:3n-9)
MEFAB	Maastricht Essential Fatty Acid Birth
MUMC	Maastricht University Medical Centre
n	number of subjects
n.a.	not applicable
NaCl	natrium (sodium) chloride
n.d.	not detectable
ObA	Osbond acid (22:5n-6)
OR	Odds Ratio
p-value	probability value
PEF	peak expiratory flow
PGE2	prostaglandin E2
PL(s)	phospholipid(s)
pm	post meridiem
PUFA(s)	polyunsaturated fatty acid(s)
R^2	coefficient of determination of total model
r^2	square of the semi-partial correlation coefficient
RAST	radioallergosorbent test
SD	standard deviation
SES	socio-economic status
SPSS	Statistical Package for the Social Sciences
STM	short-term memory
TL(s)	total lipid(s)
VAS	vibroacoustic stimulus
viz.	videlicit (that is to say)
vWF	von Willebrand factor
wt	weight

Dankwoord

Dankwoord

Na het bewandelen van een wel heel bijzonder promotietraject is het zover: mijn proefschrift is af! Ook al staat mijn naam op de kaft, zonder de hulp van anderen was dit boekje nooit tot stand gekomen. Graag wil ik dan ook alle personen bedanken die een bijdrage hebben geleverd aan dit proefschrift. Een aantal van hen wil ik hier met name noemen.

Als eerste wil ik beginnen bij mijn promotores Prof. em. dr. Gerard Hornstra en Prof. dr. Jan Nijhuis.

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List of publications

List of publications

1. Weseler AR, Dirix CE, Bruins MJ & Hornstra G. Dietary Arachidonic Acid Dose-Dependently Increases the Arachidonic Acid Concentration in Human Milk. J Nutr 2008;138:2190-2197.

2. Dirix CE, Kester AD & Hornstra G. Associations between neonatal birth dimensions and maternal essential and *trans* fatty acid contents during pregnancy and at delivery. Br J Nutr 2009;101:399-407.

3. Dirix CEH, Hogervorst JGF, Rump P, Hendriks JJE, Bruins MJ & Hornstra G. Prenatal arachidonic acid exposure and selected immune-related variables in childhood. Br J Nutr *(in press)*.

4. Dirix CEH, Nijhuis JG, Jongsma HW & Hornstra G. Aspects of fetal learning and memory. Child Dev *(in press)*.

5. Dirix CEH, Hornstra G & Nijhuis JG. Fetal learning and memory: weak associations with the early essential polyunsaturated fatty acid status. Prostaglandins Leukot Essent Fatty Acids *(in press)*.

6. Dirix CEH, Kester AD & Hornstra G. Associations between term birth dimensions and prenatal exposure to essential and *trans* fatty acids. *(Under revision)*.

Curriculum Vitae

Curriculum Vitae

Chantal Elisabeth Henricus Dirix was born in Heerlen on October the 2nd 1980, in The Netherlands. She completed secondary school at the Euro-College in Maastricht in 1999. In the same year, she started with her study Health Sciences at Maastricht University, specializing in Biological Health Sciences, and graduated in December 2003. During her internship at the Department of Human Biology at Maastricht University and the Department of Obstetrics and Gynaecology at the Academic Hospital Maastricht, she investigated the relationships between essential polyunsaturated fatty acids and fetal and maternal brain functions. During the following years she performed her PhD research at the same departments, under supervision of Prof. em. dr. G. Hornstra and Prof. dr. J.G. Nijhuis. From 2003 to 2006, she also worked as a research-assistant at NutriScience, a research organisation in Maastricht. In 2007, she was awarded with a Young Investigator grant at the scientific conference on 'Early Nutrition Programming & Health Outcomes in later life: Obesity & Beyond' in Budapest, Hungary. In 2009, she finished her thesis 'The functionality of maternal and neonatal fatty acids, *from pregnancy to childhood*'.